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QUANTITATIVE COLOUR REACTION FOR THALIDOMIDE VIA ITS HYDROLYSIS PRODUCTS BY SPECTROPHOTOMETRIC ABSORPTION TECHNIQUE

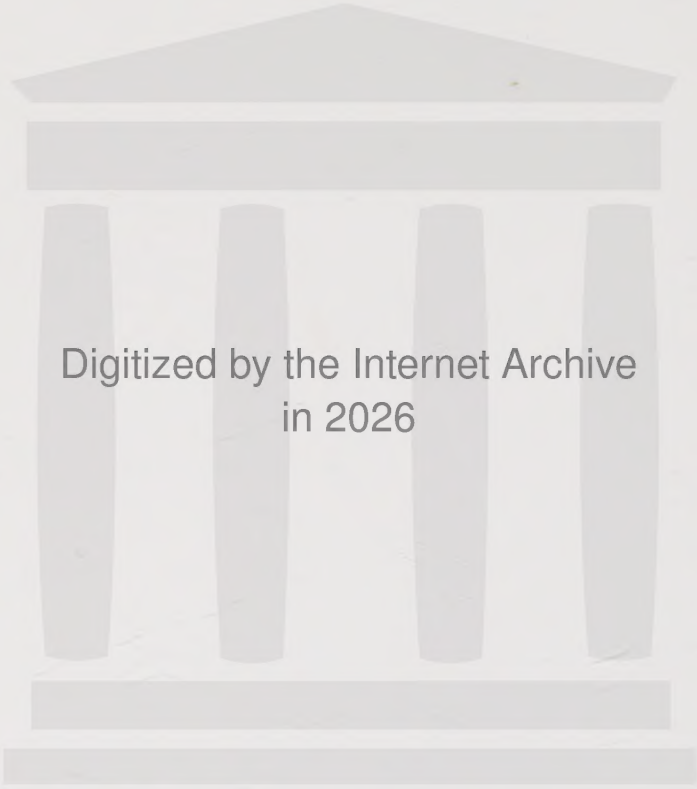
Inaugural-Dissertation
zur
Erlangung der Doktorwürde
der
Hohen Mathematisch-Naturwissenschaftlichen Fakultät
der
Rheinischen Friedrich-Wilhelms-Universität
zu
Bonn

vorgelegt von

El Sayed Lashine

aus
El Kalyobia/Ägypten

Bonn 1986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Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Universität zu Bonn.

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CONTENTS

		Page
1.	INTRODUCTION	8
2.	GENERAL PART	10
2.1.	Chemistry of Thalidomide	10
2.2.	Spontaneous Hydrolysis of Thalidomide	12
2.3.	Analytical Metabolic Chemistry of Thalidomide	13
3.	SPECIAL PART	22
3.1.	Research Objectives	22
3.2.	Method for Trace Quantities of Aldehydes	23
3.3.	Theoretical Discussion	25
3.3.1.	Direct Reduction of Carboxylic Acids to Aldehydes	25
3.3.2.	N-Methylphthalamic Acid (XIX)	30
3.3.2.1.	Reduction of N-Methylphthalamic Acid to its Aldehyde by Use of Thexylborane	31
3.3.2.2.	Reduction of N-Methylphthalamic Acid to its Aldehyde by Use of Bis(4-methylpiperazinyl)-aluminium Hydride	32
3.3.3.	α-(o-Carboxybenzamido)glutarimide (IV)	37
3.3.3.1.	Reduction of α-(o-Carboxybenzamido)glutarimide to its Aldehyde by Use of Bis(4-methyl-piperazinyl)aluminium Hydride	39
3.3.4.	2-Phthalimidoglutaramic Acid	43
3.3.4.1.	Reduction of 2-Phthalimidoglutaramic Acid to its Aldehyde by Use of Bis(4-methylpiperazinyl)-aluminium Hydride	44
3.3.5.	4-Phthalimidoglutaramic Acid	48
3.3.5.1.	Reduction of 4-Phthalimidoglutaramic Acid to its Aldehyde by Use of Bis(4-methylpiperazinyl)-aluminium Hydride	48
3.3.6.	2-Phthalimidoglutaric Acid	53

		Page
3.3.6.1.	Reduction of 2-Phthalimidoglutaric Acid to its Aldehyde by Use of Bis(4-methylpiperazinyl)-aluminium Hydride	53
3.3.7.	2-(o-Carboxybenzamido)glutaric Acid	57
3.3.7.1.	Reduction of 2-(o-Carboxybenzamido)glutaric Acid by Use of Bis(4-methylpiperazinyl)-aluminium Hydride	57
3.3.8.	Quantitative Colour Reaction of the Mixture of Thalidomide Hydrolysis Products	61
3.3.9.	Quantitative Colour Reaction of Thalidomide, Hydrolysed at pH 7.4 and 37°C for 24 Hours	66
3.3.10.	Quantitative Colour Reaction of Thalidomide, Hydrolysed at pH 7.4 and 37°C for 4 Hours	73
3.3.11.	Quantitative Colour Reaction of Thalidomide, Hydrolysed at pH 8 and 37°C for 24 Hours	77
3.3.12.	Quantitative Colour Reaction of Thalidomide, Hydrolysed at pH 8 and 37°C for 4 Hours	81
3.3.13.	Quantitative Colour Reaction of Thalidomide, Hydrolysed at pH 6 and 37°C for 24 Hours	85
3.3.14.	The Most Favourable Conditions for the Analysis of Thalidomide According to the Obtained Results	88
3.4.	Applications on Small Weight Quantities of Thalidomide, Hydrolysed at Different pH Values	90
4.	SUMMARY	92
5.	EXPERIMENTAL PART	94
5.1.	General Declaration	94
5.2.	Preparation of Reagents	95
5.2.1.	Preparation of 0.1 Mole of a 0.5 M Solution of Thexylborane in Tetrahydrofuran	95
5.2.2.	Preparation of 0.71 M Aluminium Hydride Solution	
5.2.3.	Preparation of Bis(4-methylpiperazinyl)-aluminium Hydride	96

		Page
5.2.4.	Carbonyl-Free Methanol	96
5.2.5.	2,4-Dinitrophenylhydrazine Solution	96
5.2.6.	Potassium Hydroxide Solution	97
5.2.7.	Buffer Phosphate Solution	97
5.2.8.	Absolute Tetrahydrofuran	97
5.3.	N-Methylphthalamic Acid (XIX)	98
5.3.1.	Reduction of N-Methylphthalamic Acid by Use of Thexylborane	98
5.3.2.	2,4-Dinitrophenylhydrazine Test Solution	99
5.3.3.	Reduction of N-Methylphthalamic Acid by Use of Bis(4-methylpiperazinyl)aluminium Hydride	99
5.3.4.	Quantitative Measurement of the Colour Developed from the Foregoing Hydrazone	100
5.4.	D,L,-α-Aminoglutarimide Hydrochloride (X)	103
5.4.1.	α-(o-Carboxybenzamido)glutarimide (IV)	103
5.4.2.	Reduction of α-(o-Carboxybenzamido)glutarimide by Use of Bis(4-methylpiperazinyl)aluminium Hydride	104
5.4.3.	Quantitative Measurement of the Colour Developed from the Foregoing Hydrazone	105
5.5.	Phthalyl-D,L-glutamic Anhydride	108
5.5.1.	2-Phthalimidoglutaramic Acid (III)	109
5.5.2.	Reduction of 2-Phthalimidoglutaramic Acid by Use of Bis(4-methylpiperazinyl)aluminium Hydride	109
5.5.3.	Quantitative Measurement of the Colour Developed from the Foregoing Hydrazone	110
5.6.	γ-Benzyl Hydrogen Phthalyl-D,L-Glutamate	110
5.6.1.	Phthalyl-D,L-Isoglutamine Benzyl Ester	110
5.6.2.	4-Phthalimidoglutaramic Acid (II)	111
5.6.3.	Reduction of the Dried Material of 4-Phthalimidoglutaramic Acid by Use of Bis(4-methylpiperazinyl)aluminium Hydride	111
5.6.4.	Quantitative Measurement of the Colour Developed from the Foregoing Hydrazone	112
5.7.	2-Phthalimidoglutaric Acid (V)	112

		Page
5.7.1.	Reduction of 2-Phthalimidoglutaric Acid by Use of Bis(4-methylpiperazinyl)aluminium Hydride	113
5.7.2.	Quantitative Measurement of the Colour Developed from the Foregoing Hydrazone	113
5.8.	2-(o-Carboxybenzamido)glutaric Acid (VIII)	113
5.8.1.	Reduction of 2-(o-Carboxybenzamido)glutaric Acid by Use of Bis(4-methylpiperazinyl)aluminium Hydride	114
5.8.2.	Quantitative Measurement of the Colour Developed from the Foregoing Hydrazone	114
5.9.	Reduction of a Mixture of Thalidomide Hydrolysis Products According to the Percentage Obtained by Beckmann in Table 1	119
5.9.1.	Quantitative Measurement of the Colour Developed from the Foregoing Hydrazones	119
5.10.	Reduction of Thalidomide Hydrolysis Products, Obtained after its Hydrolysis at pH 7.4 and 37°C for 24 hrs at Room Temperature	120
5.10.1.	Quantitative Measurement of the Colour Developed from the Foregoing Hydrazones	120
5.11.	Reduction of Thalidomide Hydrolysis Products, Obtained after its Hydrolysis at pH 7.4 and 37°C for 24 hrs, under Reflux	121
5.11.1.	Quantitative Measurement of the Colour Developed from the Foregoing Hydrazones	121
5.12.	Reduction of Thalidomide Hydrolysis Products, Obtained after its Hydrolysis at pH 7.4 and 37°C for 4 hrs, under Reflux	122
5.12.1.	Quantitative Measurement of the Colour Developed from the Foregoing Hydrazones	122
5.13.	Reduction of Thalidomide Hydrolysis Products, Obtained after its Hydrolysis at pH 8 and 37°C for 24 hrs, under Reflux	123
5.13.1.	Quantitative Measurement of the Colour Developed from the Foregoing Hydrazones	123

Page

5.14. Reduction of Thalidomide Hydrolysis Products, Obtained after its Hydrolysis at pH 8 and 37°C for 4 hrs, under Reflux 124

5.14.1. Quantitative Measurement of the Colour Developed from the Foregoing Hydrazones 124

5.15. Reduction of Thalidomide Hydrolysis Products, Obtained after its Hydrolysis at pH 6 and 37°C for 24 hrs, under Reflux 125

5.15.1. Quantitative Measurement of the Colour Developed from the Foregoing Hydrazones 125

5.16. Applications on Small Weight Quantities of Thalidomide Hydrolysed at Different pH Values and 37°C for Different Times 126

5.16.1. Method for Unknown Trace Quantities of Aldehydes 126

6. REFERENCES 128

1. INTRODUCTION

Thalidomide, which has earned a dubious fame after the Contergan-tragedy, is occurently being tested with partial success against various skin diseases. Already in the mid sixties, this substance was used in the treatment of scurf. Meanwhile, its effectiveness against leprosy seems to be established. It also seems to be effective against chronic skin tuberculosis and pruritis. How thalidomide works in each of these different cases is not yet clear. Women who have conceived or are pregnant should never be treated with thalidomide [1]. Thus the chapter on thalidomide has not come to an end.

Thalidomide was first synthesized in Germany in 1956 by King et al. [2], who also described its main pharmacological properties. It was found to be a nontoxic sedative hypnotic drug with quietening effect upon the central nervous system, reducing the voluntary activity of laboratory animals and promoting sleep. Its ability to promote sleep in man was shown in 1956 by Jung [3] and in 1960, there was apparently convincing evidence of its nontoxicity. However, late in 1960, the first reports [4] of a toxic effect due to thalidomide began to appear. These were cases of peripheral neuritis following regular medication with the drug [5].

---

1) Anon.: Dt.Apoth.Zeitg. 19, 925 (1983).
2) W.King, H.Keller and H.Muckter: Arzneim.Forsch. 6, 426 (1956).
3) H.Jung: Arzneim.Forsch. 6, 430 (1956).
4) A.L.Florence: Brit.Med.J. 2, 1954 (1960).
5) P.M.Fullerton and M.Kremer: Brit.Med.J. 2, 855 (1961).

The symptoms developed in two to eighteen months after starting regular medication. The occurrence of the condition was random but recovery on withdrawal of the drug was slow except mild cases. However, the peripheral neuritis produced by thalidomide was not considered to be very serious, at least in Europe, since only a small proportion of the users of the drug appeared to be affected.

Late in 1961, a paper was published in Germany by Lenz [6] who suggested that thalidomide was responsible for limb deformations in newborn infants. This suggestion was confirmed by others in a number of papers published in 1962 in British and German journals [7-14].

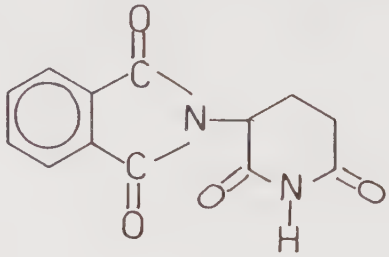
---

6) W.Lenz: Deutsch.Med.Wschr. 86, 2555 (1961).
7) R.Beckmann: Arzneim.Forsch. 12, 1095 (1962).
8) F.Kemper: Arzneim.Forsch. 12, 640 (1962).
9) F.G.Somers: Lancet I, 912 (1962).
10) G.Pliess: Lancet I, 1128 (1962).
11) A.Giroud, H.Tauchmann-Duplessis and L.Mercier-Parot: Lancet II, 298 (1962).
12) G.A.Christie: Lancet II, 249 (1962).
13) D.Felisati: Lancet II, 742 (1962).
14) J.M.Seller: Lancet II, 249 (1962).

2. GENERAL PART

2.1. Chemistry of Thalidomide

Chemically thalidomide (I) is (±)-α-phthalimidoglutarimide, or more fully, 1,3-dioxo-2-(2',6'-dioxopiperidin-3'-yl)isoindoline. It is made up of two parts, the phthalimide structure, which is responsible for the toxicological action, and the glutarimide ring which is responsible for the pharmacological action [15].



(I)

It is a white crystallin solid, melting point 271°C, which is sparingly soluble in water (6 mg/100 ml), dilute hydrochloric acid, benzene and ether, but highly soluble in acetone, dimethylformamide and dioxan. Its low solubility in water suggests that its concentration in body fluids at any time would be small. The sparing solubility of thalidomide in water led several workers to attempt to make strong aqueous solution of it by dissolving it in dilute alkali and then neutralizing the solution. But, as will be shown later, such solutions do not contain thalidomide, since the drug is immediately decomposed by dissolution in alkali.

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15) H.Koch: Sci.Pharm. 49, 47 (1981).

There are two important features of the thalidomide molecule to which attention should be drawn. These are the asymmetric carbon atom at position 3' of the glutarimide ring and the two imide functions with the four substituted amide bonds which are located at 1-2 and 2-3 in the phthalimide ring and 1'-2' and 1'-6' in the glutarimide ring. The drug is the optically inactive (±)- form. The (+)- and (-)-forms of the drug have been made and are more soluble (25 mg/100 ml) in water than the (±)-form. Although they are both of low toxicity, the (-)-form has a lower LD_{50} in mice than the (±)- and (+)-forms. The optically active forms of thalidomide are not easy to obtain, but limited experiments suggest that the (-)-form may be less teratogenic [17]. However new datas show that only the S-(-)-enantiomer is teratogenic [18], and the R-(+)-enantiomer is uneffective even in high doses. These new data completed the earlier results which show that the S-(-)-enantiomer of 2-phthalimidoglutaric acid (V) was active [19,20].

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16) R.L.Smith, S.Fabro, H.Schumacher and R.T.Williams: in: "Embryopathic Activity of Drugs", J.M.Robson, F.Sullivan, R.L.Smith (Eds.), J.& A.Churchil Ltd. London, 194 (1965).

17) S.Fabro, R.L.Smith and R.T.Williams: "Toxicity and Teratogenicity of Optical Isomers of Thalidomide", Nature, 215 (1967).

18) K.Fickentscher, F.Köhler, G.Blaschke and H.P.Kraft: Arzneim.Forsch. 29, 1640 (1979).

19) H.Ockenfels and F.Köhler: Experientia 26, 1236 (1970).

20) H.Ockenfels, F.Köhler and W.Meise: Pharmazie 31, 492 (1976).

On the other hand, many work was done to test the teratogenic activity of the hydrolysis products of thalidomide [21-25]. The interesting result was to find that α-(o-carboxybenzamido)-glutarimide and D,L-α-aminoglutarimide hydrochloride did not lead to fetal malformations after i.p. administration to pregnant mice [26]. Thus of the twelve hydrolysis products of thalidomide only those three which contain the phthalimide moity show teratogenic activity, i.e. 4-phthalimidoglutaramic acid (II), 2-phthalimidoglutaramic acid (III) and 2-phthalimidoglutaric acid (V).

2.2. Spontaneous Hydrolysis of Thalidomide

The two bonds in the phthalimide ring are equivalent, but the two bonds of the glutarimide ring are different, for the splitting of the 1'-2' bond gives rise to glutamine derivatives, whereas splitting of the 1'-6' bond gives rise to isoglutamine derivatives [27] Fig.1.

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21) F.Köhler and H.Ockenfels: Experientia 26, 1157 (1970).
22) F.Köhler and W.Meise: Z.Naturforsch. 26b, 857 (1971).
23) W.Meise and F.Köhler: Z.Naturforsch. 26b, 1081 (1971).
24) F.Köhler, W.Meise and H.Ockenfels: Experientia 27, 1149 (1972).
25) W.Meise and F.Köhler: Pharmazie 27, 418 (1972).
26) W.Meise, H.Ockenfels and F.Köhler: Experientia 29, 423 (1973).
27) R.T.Williams: Arch.Environ.Health 16, 493 (1968).

Earlier studies on the nature of the urinary extractory products of thalidomide in man, dog, rat and rabbit showed that these products were those that would be formed if the drug was hydrolysed at its amide bonds. These products could be formed enzymatically or spontaneously. This led to investigate the stability of thalidomide at physiological pH values, and it was found, that thalidomide was unstable in aqueous solution, and in fact, after storage of an aqueous solution of the drug, it gave rise to all twelve possible products [27].

2.3. Analytical Metabolic Chemistry of Thalidomide

The first investigation on the metabolism of this sedative originate in the laboratory of its development of the firm Grünenthal.

In 1962 Beckmann [7] described the synthesis of a labelled thalidomide from 7-^{14}C-labelled phthalic anhydride. After administration of this compound, paper chromatography revealed only traces of unchanged drug and ß-glucuronidase hydrolysis gave no evidence of conjugation derivatives.

Beckmann synthesized [28] a series of derivatives to allow paper chromatographic identification. ß-Glucuronidase hydrolysis did not modify the chromatograms. Hydrolysis in situ with sodium rhobizonate detection for sulphate conjugation was negative. The amounts of each metabolite were determined by reverse isotopic dilution.
The reference compound was dissolved in alkalinized urine, precipitated with the corresponding metabolite by acidification, and crystallized at 4°C. The cycle was repeated until constant activity was achieved.

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7) R.Beckmann: Arzneim.Forsch. 12, 1095 (1962).
28) R.Beckmann: Arzneim.Forsch. 13, 185 (1963).

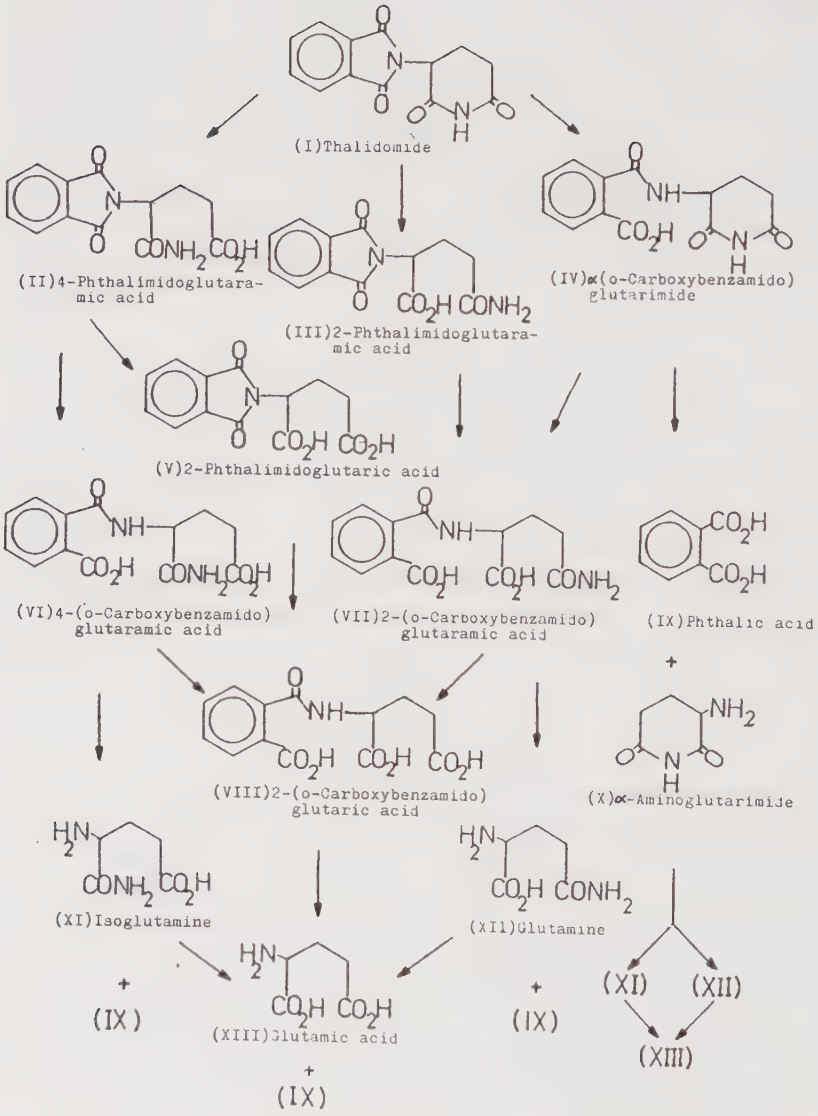


Fig. 1: Hydrolysis Products of Thalidomide

Larger amounts of some metabolites were obtained by column chromatography on Amberlite IR-4B and elution by formic acid solution of increasing concentrations from 0.001N to 4N. The metabolites thus separated were identified by their retention volume, their R_f on paper, their melting points and their IR-spectra. The values of these metabolites occuring in urine are shown in table 1 [29,30].

Table 1

Compound found	As % of urinary ^{14}C in man	rat	dog
α-(o-carboxybenzamido)-glutarimide	29.5	35.54	22.9
4-phthalimidoglutaramic acid	53.2	7.14	2.2
2-phthalimidoglutaramic acid	5.0	8.16	13.6
2-phthalimidoglutaric acid	3.5	6.15	9.0
4-(o-carboxybenzamido)glutaramic acid 2-(o-carboxybenzamido)glutaramic acid	....	5.13	....
2-(o-carboxybenzamido)glutaric acid	2.3	2.4	4.7
phthalic acid	2.7	2.9	6.1
thalidomide	1.0	2.2	1.8

29) J.W.Faigle, H.Kæberle, W.Riess and K.Schmid: Experientia 18, 389 (1962).

30) R.D.Mackensie and W.R.McGrath; Proc.Soc.Exp.Biol.Med. 109, 511 (1962).

At the same time, Mackenzie and McGrath [30] also administered labelled thalidomide to rats and examined urine and organ extracts by paper chromatography. The identity of the metabolites was confirmed by reverse isotopic dilution.

Faigle et al. [29] prepared a thalidomide ^{14}C-labelled on the two phthalimide carbonyls. They demonstrated that 70% of the drug were excreted unchanged in the faeces of the dogs, as shown by isotopic dilution. The residual 30% were metabolites in urine. These were identified after synthesis of the authentic compounds, by paper and thin-layer chromatography and by counter-current distribution. Their concentration was determined by revers isotopic dilution.

Smith et al. [31] characterized one of the metabolites of thalidomide as phthaloylisoglutamine. It was extracted from urine by acetone and crystallized on concentration. It was identified by comparison with authentic compounds and it was possible to separate it from phthaloylglutamine by electrophoresis.

In 1964-1965, the Schumacher, Smith and Williams team published a series of papers [32,33] on the hydrolysis of thalidomide at various pH values. They elaborated a paper chromatographic method derived from Beckmann.

---

31) R.L.Smith, R.A.D.Williams and R.T.Williams: Life Sci. 1, 333 (1962).

32) H.Schumacher, R.L.Smith, R.B.Stagg and R.T.Williams: Pharm. Acta Helv. 39, 394 (1964).

33) H.Schumacher, R.L.Smith and R.T.Williams: Brit.J.Pharmacol. 25, 324 (1965).

The effect of pH on hydrolysis of thalidomide and the amount of hydrolysis products are shown in Table 2. They subsequently [34] fractionated the urinary metabolites by administration of unlabelled drug. The metablites are isolated by two procedures. The first was by column chromatography on alumina of strongly concentrated urine. The eluting solvent was the system benzene:chloroform 3:1, to which were added increasing amounts of a mixture of dioxan, methylethylketone, acetone, methanol and chloroform equal parts. The various fractions were examined by paper chromatography. Some were analysed by alumina thin-layer chromatography for preparative purposes. The second fractionation procedure involved a series of extractions and crystallyzation and is summarized in Fig. 2, p. 21. The quantitative identifications were completed by colour reactions, IR spectra, melting points and elementary analysis.

Table 2

Compound at p. 14	Amount of hydrolysis products formed (%) at					
	pH 6		pH 7.4		pH 8	
	4 hrs	24 hrs	4 hrs	24 hrs	4 hrs	24 hrs
(I)	84.0	58.3	41.5	0.15	3.2	0.03
(II)	2.5	4.9	3.5	2.0	3.7	3.3
(III)	0	1.3	1.1	1.8	2.8	6.2
(IV)	12.7	34.5	47.5	52.5	58.0	14.0
(V)	0	0	0	0.1	1.0	2.7
(VI)	0	0.7	1.8	23.0	18.0	40.5
(VII)	0	0	1.6	20.0	10.0	25.7
(VIII)	0	0	0	0.1	0.5	5.2
(IX)	0	0	0	0.1	1.4	1.9

34) H.Schumacher, R.L.Smith and R.T.Williams: Brit.J.Pharmacol. 25, 338 (1965).

Fabro et al. [35] used a ^{14}C-labelled thalidomide and were able to interpret the metabolism quantitatively.

Paper chromatography enabled Nicholls [36] to study the transfer of labelled thalidomide and its metabolites from the mother to the foetus. This author used the mixture isopropanol:water 4:1 but did not report the R_f values. The data pertinent to paper chromatography of thalidomide metabolites are collected in Table 3.

Table 3

Compound at p. 14	R_f in system: [a] 1	2	3	4a	4b	5
(I)	0.80	0.80	0.77	0.90	0.89	tailing
(II)			0.75	0.45	0.47	0.63
(III)			0.72	0.41	0.37	0.59
(IV)			0.60	0.40	0.38	0.63
(V)			0.81	0.22	0.26	0.83
(VI)			0.58	0.17	0.16	0.47
(VII)	0.80	1.00	0.57	0.16	0.17	0.50
(VIII)	0.80	0.04	0.64	0.10	0.18	0.46
(IX)	0.86	0.20	0.71	0.52	0.57	0.74
(X)					0.41	0.12
(XI)					0.13	0.13
(XII)					0.10	0.05
(XIII)					0.06	0.09

[a] Solvent system: 1) dimethylformamide:methanol:water 25:70:5 [30]; 2) 2% ammoniumhydroxide saturated butanol [30]; 3) butanol:acetic acid:water 4:4:1 [28]; 4) pyridine:amylalcohol:water 7:7:6; 4a [28], 4b [33], 5) butanol:acetic acid 10:1 water saturated [33]. Bidimensional chromatography: solvent 4, then solvent 5 [33,34].

35) S.Fabro, H.Schumacher, R.L.Smith and R.T.Williams: Nature 201, 1125 (1964).

36) P.J.Nicholls: J.Pharm.Pharmacol. 18, 46 (1966).

A new method for the separation of thalidomide and its metabolites by thin-layer chromatography was described by Koch et al. [37]. The most favourable results were obtained on layers of kieselgel G and GF_{254} respectively with various solvent systems. The R_f values of thalidomide and its hydrolysis products are given in Table 4.

Table 4

Compound at p.14	R_f value x 100 syst.I	syst.II	syst.III	syst.IV	syst.V	detection a	b	c	d	e
(I)	81	63	35	71	63	+	–	–	+	+
(II)	50	25	12	50	44	+	+	–	+	+
(III)	45	22	10	54	41	+	+	–	+	+
(IV)	35	13	8		33	–	–	+	+	+
(V)	74	46	25	42	29	+	+	–	+	+
(VI)	25	7	5		14	–	+	–	+	+
(VII)	18	4	0		12	+	+	+	+	+
(VIII)	42	11	3		16	+	+	+	+	+
(IX)	64	37	27	60	38	–	+	–	–	+
(X)	0	0	0	51	4	–	–	+	–	+
(XI)	0	0	0	29	23	–	–	+	–	–
(XII)	0	0	0	33	25	–	–	+	–	–
(XIII)	0	0	0	19	10	–	–	+	–	–

System I chloroform:isopropanol:formic acid 75:20:5
System II chloroform:isopropanol:formic acid 85:10:5
System III benzene:dioxan:formic acid 75:20:5
System IV collidine:water 75:25
System V pyridine:amyl alcohol:water 35:35:30

Detection: a) 5% iodine chloroform solution; b) bromocresol green spray; c) ninhydrin spray; d) Fe-hydroxylamine [28,38]; e) fluorescence solution at 254 nm.

37) G.Pischek, E.Kaiser and H.Koch: Mikrochim. Acta 530 (1970).
38) M.Frahm, A.Gottesleben and K.Soehring: Pharm. Acta Helv. 38, 785 (1963).

An ultraviolet spectrophotometric method was described by Green and Benson [39] for the determination of thalidomide in blood, plasma and urine. Thalidomide was extracted from the sample with chloroform, and the drop in extinction measured when the solutions are treated with alkali. The method has been used to determine the amount of the drug present in animal and human blood.

Similar work was described by Beckmann and Kampf [40]. The extinction of thalidomide was measured at 220 nm and 300 nm, and after addition of alkali. The band at 220 nm disappeared and a hypsochromic shift occured for the band at 300 nm with low extinction value.

39) J.N.Green and B.C.Benson: J.Pharm.Pharmacol. 13, 117 (1961).

40) R.Beckmann and H.H.Kampf: Arzneim.Forsch.11, 45 (1961).

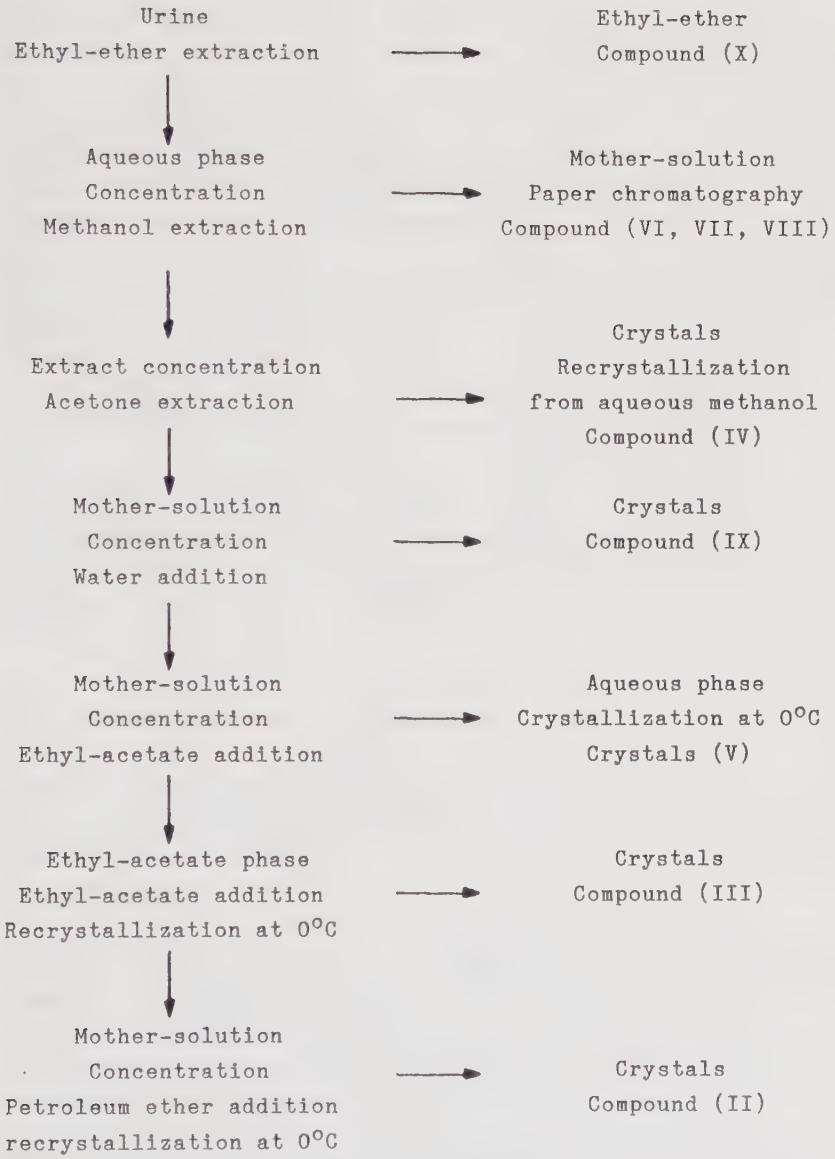
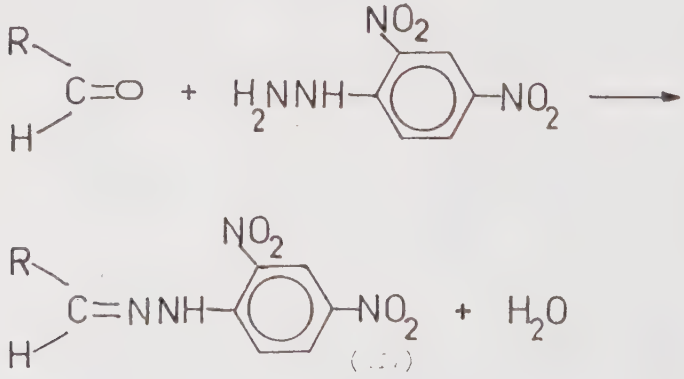


Fig. 2: Fractionation of thalidomide urinary metabolites after Schumacher [33].

3. SPECIAL PART

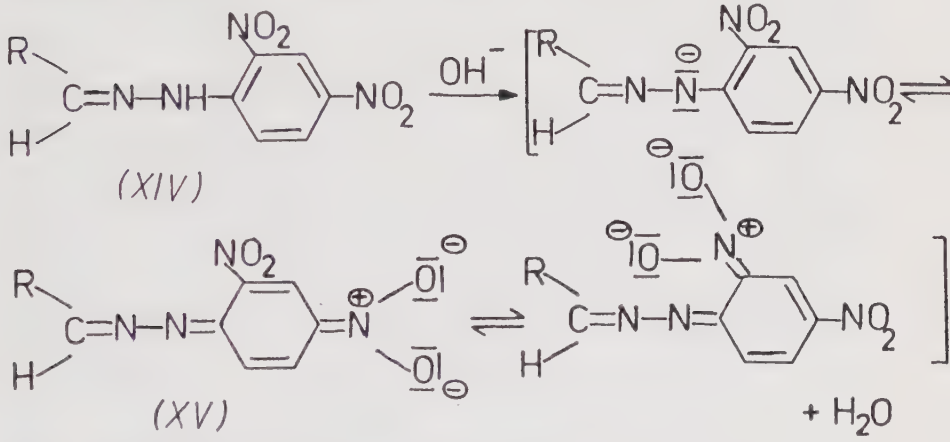
3.1. Research Objectives

An accurate gravimetric analysis of aldehydes is based on their conversion to the corresponding 2,4-dinitrophenylhydrazones. These aldehydes are obtained by selective direct reduction of the carboxylic acids, which are the hydrolysis products of thalidomide. The reduction procedure is carried out on the main hydrolysis products of thalidomide separately after their synthesis, or in mixture after the hydrolysis of thalidomide at different pH values using phosphate buffer. 2,4-dinitrophenylhydrazones are insoluble, coloured crystalline derivatives. They are easily isolated and weighed, and the high molecular weight of the hydrazones permits the analysis of relatively small samples. A sample containing about $4x10^{-4}$ moles of aldehyde is adequate for analysis [41]. The hydrazone formation is carried out in acid solution and the mixture is filtered through a tared filter, which is then weighed. This method is quite specific with most classes of compounds presenting no interference.



41.) S.Siggia: "Quantitative Organic Analysis via Functional Groups", 4th ed., John Wiley & Sons, New York, Chap.2 (1979).

When alcoholic alkali is added to a 2,4-dinitrophenylhydrazone (XIV), a blue colour is produced, probably as a result of resonance delocalization of the negative charge resulting from removal of a proton, presumably because of the formation of the resonating quinoidal ion (XV). This colour provides a very sensitive spectrophotometric assay for aldehydes.



(XIV)

(XV)

3.2. Method for Trace Quantities of Aldehydes

Traces of aldehydes can often be determined by ultraviolet absorption techniques [42], especially the compounds where the carbonyl double bond is in conjunction with carbon-to-carbon double bonds.

Also polarography can be used for determining trace quantities of aldehydes. But traces of carbonyl material as a class of compounds are very amenable to colourimetric determination, since they undergo quantitative reactions with coloured products. The colour reaction the most widely used for this purpose is the 2,4-dinitrophenylhydrazone formation. The quantitative determination of traces of aldehyde can be carried out in water, organic solvents or organic reaction products.

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42) G.R.Lippin and L.C.Clark: Anal.Chem. 23, 541 (1951).

The method is useful in range of aldehyde concentrations from 10^{-4} to 10^{-6} M, whereas few if any other methods give reliable results or are of general application.

Absorption spectra are run on alkaline alcoholic solutions of a number of 2,4-dinitrophenylhydrazones. The position of absorption as well as the value of E_{max} are nearly independent of the concentration of base as long as a sufficient excess is present.

For actual analysis it is unnecessary to isolate the hydrazone. It is prepared in solution using an excess of 2,4-dinitrophenylhydrazin, the addition of base converts the excess reagent to a very light yellow substance, the absorption of which is corrected by using a blank determination.

In this work the gravimetric analysis is carried out beside the spectrophotometric analysis, in order to plan a calibration curve relating the absorption of colour to the amount of the hydrazone producing this colour. It is related to the amount of the parent compound. From the planned calibration curve and its regression equation, any traces of unknown compounds can be determined using the method for trace quantities of aldehydes.

$$y = mx + b$$

$$m = \frac{\Sigma xy - \frac{\Sigma x \cdot \Sigma y}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$$

$$b = y' - mx'$$

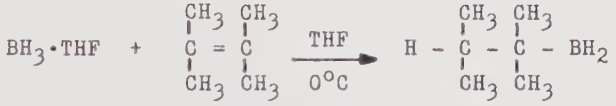
y = Absorption
m = Slope
x = Concentration
b = Intersect
y' = the Mean Absorption
x' = the Mean Concentration

3.3. Theoretical Discussion

3.3.1. Direct Reduction of Carboxylic Acids to Aldehydes

Thexylborane [43] 2,3-dimethyl-2-butylborane was the first reagent, which has been used in this work as a selective reducing agent for carboxylic acids to aldehydes. The guide carboxylic acid for this reduction was the N-methyl-phthalamic acid, because this acid contains a secondary amidic group ortho to a carboxylic group, as in case of α-(o-carboxybenzamido)glutarimide, which is one of the main hydrolysis products of thalidomide.

Thexylborane (XVI) has been prepared [44,45] by the hydroboration of 2,3-dimethyl-2-butene with borane in ratio 1:1. The solution of thexylborane in tetrahydrofuran is stable at 0°C for about one week, when stored and handled under dry nitrogen or argon.



(XVI)

Long term storage of the reagent presents no hazard and the total hydride concentration remains essentially constant. However, the bulky thexyl group undergoes slow isomerisation. Consequently, it is strongly recommended that the reagent is stored at 0°C and used within one week of preparation [43].

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43) H.C.Brown, P.Hein and N.M.Yoon: J.Org.Chem. 37, 2942 (1972).

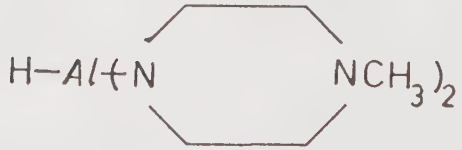
44) H.C.Brown and A.W.Moerikofer: J.Am.Chem.Soc. 84, 1478 (1962).

45) H.C.Brown and G.J.Klender: Inorg.Chem. 1, 204 (1962).

As will be mentioned, the reduction of N-methylphthalamic acid as well as the hydrolysis products of thalidomide with thexylborane gave very low yields of aldehydes as analysed gravimetrically with 2,4-dinitrophenylhydrazine.

Many methods have been reported for the preparation of aldehydes from carboxylic acid derivatives such as esters, amides and halides. However, little work has been reported on the selective reduction of free carboxylic acids to aldehydes except that by use of lithium in methylamine [46] and thexylborane [43].

A new method for the preparation of aldehydes from carboxylic acids in high yields by use of diaminoaluminium hydride is described [47]. It was found that bis(4-methylpiperazinyl)-aluminium hydride (XVII) is the most suitable reducing agent among the diaminoaluminium hydrides, which reduces carboxylic acids to the corresponding aldehydes at room temperature or under reflux in good yields.



(XVII)

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46) A.O.Bedenbaugh, J.H.Bedenbaugh, W.A.Bergin and J.D.Adkins: J.Am.Chem.Soc. 92, 5774 (1970).

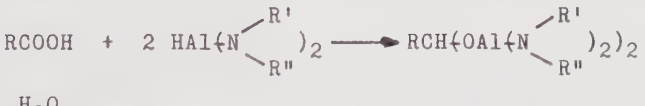
47) M.Muraki and T.Mukaiyama: Chem.Lett. 1447 (1974).

Recently, it was shown in reviews by Malek [48] and by Rerick [49], that lithium alkoxyaluminium hydride prepared from lithium aluminium hydrides and alcohols are mild reducing agents which make the reduction of carboxylic acid derivatives to aldehydes possible .
However, the reduction of carboxylic acids with lithium trimethoxyaluminium hydride [50] or aluminium hydride [51] in stoichiometric ratio gave alcohols and, if any, only trace amounts of aldehydes. No reaction took place when lithium tri-t-butoxyaluminium hydride was used in the reduction of carboxylic acids.

Aluminium hydride in tetrahydrofuran reacts readily with two moles of secondary amines such as N-methylpiperazine, morpholine, di-n-propylamine, N-methylaniline and form at room temperature with liberation of two moles of hydrogen (XVIII).

(XVIII)

Treatment of a mixture of one mole of carboxylic acid with two moles of the reagent in tetrahydrofuran either stirring 1-2 days at room temperature or refluxing for several hours, followed by hydrolysis afforded the corresponding aldehyde.



48) J.Malek and M.Gerny: Synthesis 217 (1972).
49) M.N.Perick: "Reduction: Techniques and Applications in Organic Synthesis", ed. by R.L.Augustine, Marcel Dekker, Inc., New York, Chap. 1 (1968).
50) H.C.Brown and P.M.Weissman: J.Am.Chem.Soc. 87, 5624 (1965).
51) H.C.Brown and N.M.Yoon: ibid. 88, 1464 (1966).

Aluminium hydride may be prepared by treating lithium aluminium hydride with aluminium chloride [52], the lithium chloride precipitates and an ether solution of the reagent is obtained.

$$3\ LiAlH_4 + AlCl_3 \xrightarrow{ether} 4\ AlH_3 + 3\ LiCl$$

Unfortunately, such solutions are metastable and, on standing, the aluminium hydride associates and precipitates from the solution [52,53]. This behaviour has discouraged any significant study of its reducing characteristics [54,55].

$$x\ AlH_3 \longrightarrow (AlH_3)_x$$

This tendency has led to utilize solutions stabilized by the presence of additional aluminium chloride [56-59]. Such solutions doubtless contain intermediates like AlH_2Cl, $AlHCl_2$ [56-59], and their addition compounds with lithium chloride (lithium chloride does not precipitate from these solutions containing aluminium chloride in excess over the amount required to form aluminium hydride) [59].

In view of this background, Brown [51] decided to undertake the development of a simple, convient procedure for the preparation of aluminium hydride.

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52) A.E.Finholt, A.C.Bond and H.I.Schlesinger: J.Am.Chem.Soc. 69, 199 (1952).
53) E.Wiberg, H.Graf, M.Schmidt and R.Uson: Z.Naturforsch. 7b, 578 (1952).
54) E.Wiberg and A.Jahn: ibid. 7b, 581 (1952).
55) M.J.Jorgenson: Tetrahedron Lett. 13, 559 (1962).
56) E.Wiberg: Angew.Chem. 65, 16 (1953).
57) R.F.Nystrom: J.Am.Chem.Soc. 77, 2544 (1955).
58) J.H.Brewster and H.O.Bayer: J.Org.Chem. 29, 105 (1964).
59) E.E.Eliel: Rec.Chem.Prog. 22, 129 (1961).

The procedure finally adopted by Brown, which has been used in this work, involved the addition of 0.5 moles of 100% sulfuric acid to 1 mole of lithium aluminium hydride in tetrahydrofuran solution. Hydrogen was evolved and lithum sulphate precipitated quantitatively.

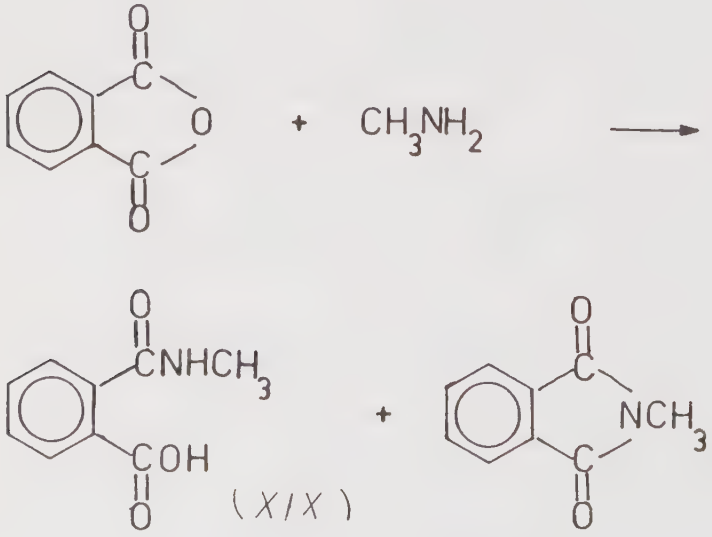
$$2\ LiAlH_4 + H_2SO_4 \xrightarrow{THF} Li_2SO_4 + 2\ AlH_3 + 2\ H_2$$

The lithium sulphate separated easily to give a clear supernatant solution. However, the solution prepared by this procedure proved to be adequately stable at room temperature for ones need.

3.3.2. N-Methylphthalamic Acid (XIX)

This compound was prepared as a guide carboxylic acid, in order to adjust the conditions of selective reduction of carboxylic acids to aldehydes, because of the similarity in structure between N-methylphthalamic acid and α-(o-caboxybenzamido)glutarimide, which is one of the main hydrolysis products of thalidomide (each of them contains a carboxylic group, ortho to a secondary amide).

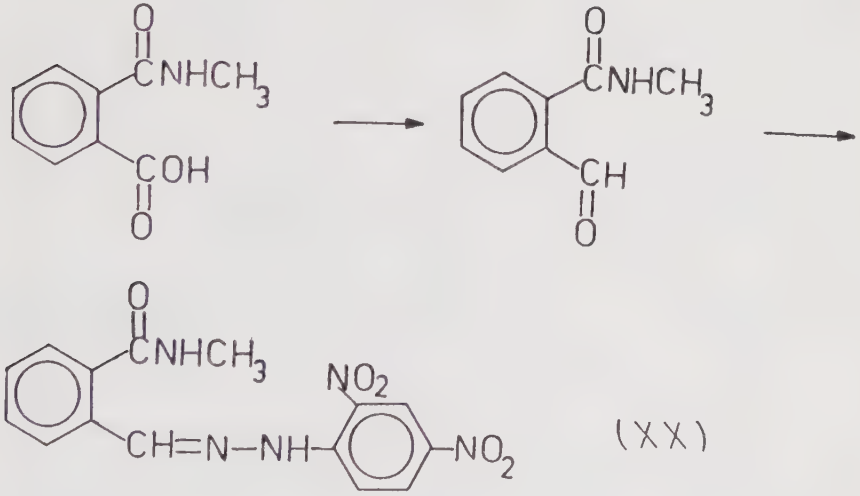
N-methylphthalamic acid was prepared [60] by the action of aqueous methylamine on phthalic anhydride. The solution was acidified with hydrochloric acid and N-mehtylphthalamic acid was separated from N-methylphthalimide by dissolving it in sodium hydroxide. The solution was then acidified with hydrochloric acid to give (XIX).



60) F.S.Spring and J.C.Woods: J.Chem.Soc. 625 (1945).

3.3.2.1. Reduction of N-Methylphthalamic Acid to its Aldehyde by Use of Thexylborane

Many attempts were carried out to reduce N-methylphthalamic acid to the corresponding aldehyde by use of thexylborane as selective reducing agent of carboxylic acids to aldehydes. The reduction was carried out under reflux and the reaction mixture was analysed with 2,4-dinitrophenylhydrazine for the aldehyde after time intervals until the yield of 2,4-dinitrophenylhydrazone remained constant.



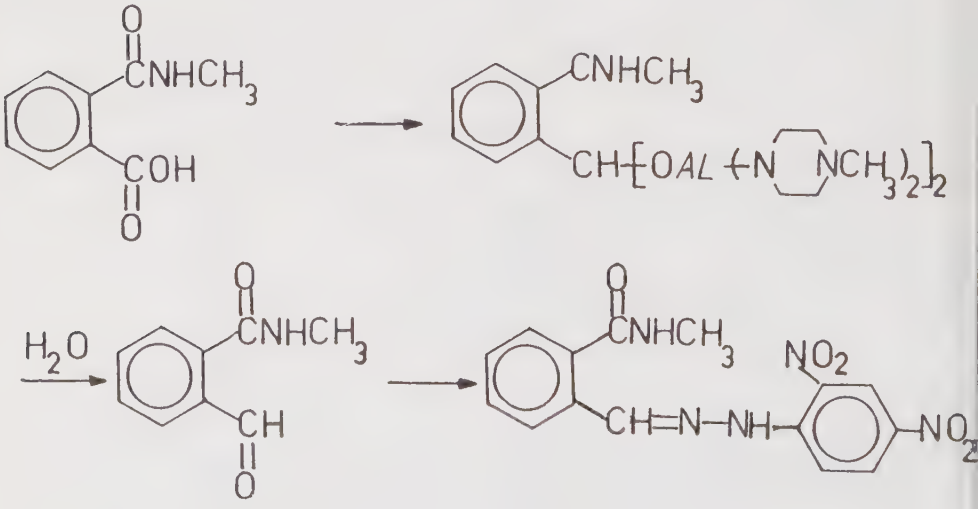
(XX)

Unexpected as it was, the yield of the above 2,4-dinitrophenylhydrazone (XX) was very low, after the reduction by use of thexylborane. Infrared spectroscopy, ^{1}H.n.m.r. spectroscopy and mass spectroscopy confirm the above 2,4-dinitrophenylhydrazone structure.

Furthermore, the reduction of the hydrolysis products of thalidomide by use of thexylborane was unsuccessful. This result has discouraged any significant study on the hydrazone, which was obtained after the reduction of N-methylphthalamic acid by use of thexylborane.

3.3.2.2. Reduction of N-Methylphthalamic Acid to its Aldehyde by Use of Bis(4-methylpiperazinyl)aluminium Hydride

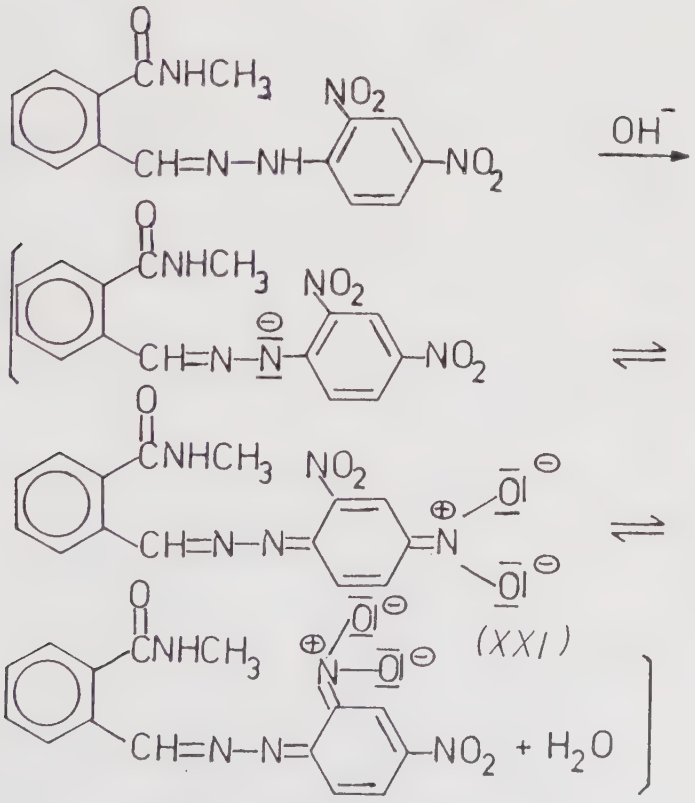
Because of the discouraging results of the reduction by use of thexylborane, it was found that bis(4-methylpiperazinyl)-aluminium hydride is the most suitable reducing agent of carboxylic acids to aldehydes. N-methylphthalamic acid was reduced to the corresponding aldehyde at room temperature or under reflux in a very good yield by use of bis(4-methyl-piperazinyl)aluminium hydride as selective reducing agent.The reduction under reflux was carried out for 6-14 hours and it was found, that refluxing for 12 hours gave the most favour-able yield of aldehyde, as analysed gravimetrically with 2,4-dinitrophenylhydrazine after hydrolysis with water to obtain the free aldehyde.



Infrared spectroscopy, ^{1}H.n.m.r. spectroscopy, mass spectroscopy and microanalysis confirm the above assigned structure of the hydrazone (XX).

When methanolic potassium hydroxide was added to the above obtained 2,4-dinitrophenylhydrazone, it gave an intense blue colour with absorption maximum at 612 nm. The relationship between the absorbance at 612 nm and the concentration of the 2,4-dinitrophenylhydrazone is linear and Beer's law is followed.

The development of the blue colour by addition of methanolic potassium hydroxide is probably a result of the resonance delocalization of the negative charge resulting from the removal of a proton and presumably because of the formation of the resonating quinoidal ion (XXI).



If the absorbance of a series of solutions of the same hydrazone is measured at the same wavelength, temperature and solvent conditions, and the absorbance of each solution is plotted against its concentration, a straight line passing the origin will usually be observed. This graph is called a Beer's law plot, and if the line is straight Beer's law is said to be obeyed over the concentration range investigated. Figure 3 shows a Beer's law study of the hydrazone, obtained from N-methylphthalamic acid after its reduction to the aldehyde. Table 5 gives the data with which Fig. 4 was plotted. Evidently Beer's law is obeyed by this system. The wavelength selected for the absorptivity determination is usually λ_{max} for two reasons:

(a) maximum sensitivity is achieved by working at the band maximum, since a given concentration produces the strongest signal at this wavelength;

(b) the change of absorbance with small change in wavelength is minimal at the band maximum unless the band is extremely sharp.

Therefore minor errors in setting the instrument's wavelength selector dial do not cause serious errors in the absorbance measurement.

The unknown concentration may be read directly from the Beer's law plot. This method is successful.

Table 5

Concentration µg/25ml	Absorption at 229 nm	Absorption at 265 nm	Absorption at 426 nm	Absorption at 612 nm
207.5	0.2302	0.2672	0.0882	0.1866
415.0	0.7727	0.5343	0.1763	0.4114
622.5	1.1940	0.8104	0.2690	0.6150
830.0	1.6130	1.0930	0.3660	0.8210
1037.5	2.0000	1.3400	0.4500	1.0040

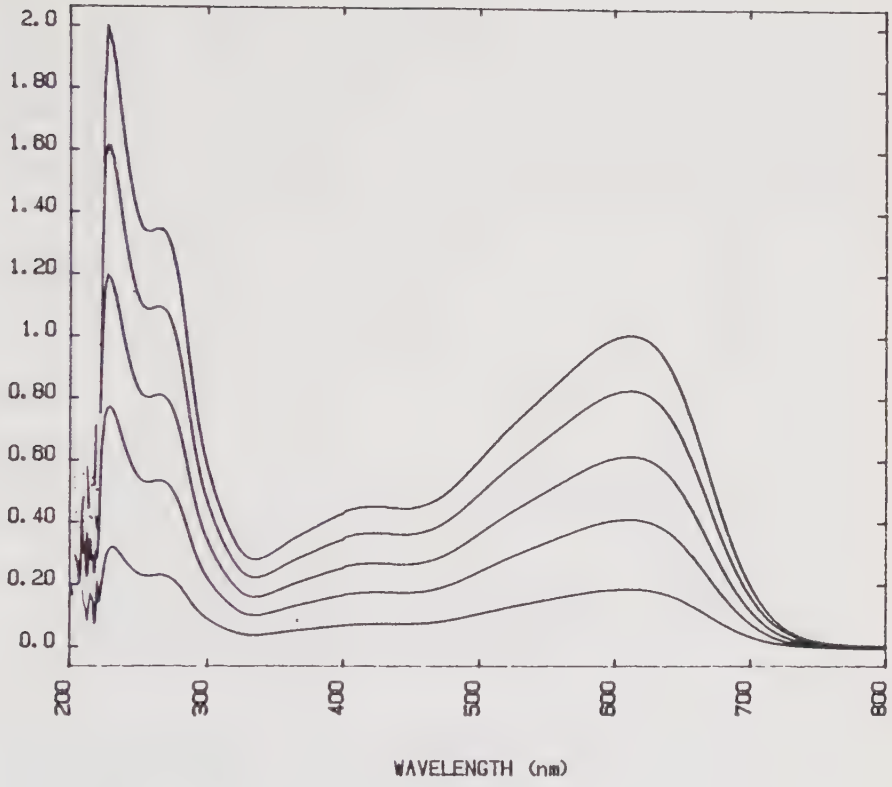


Fig.3: Absorption spectra of five solutions of the hydrazone obtained from N-methylphthalamic aldehyde in methanolic potassium hydroxide solution.

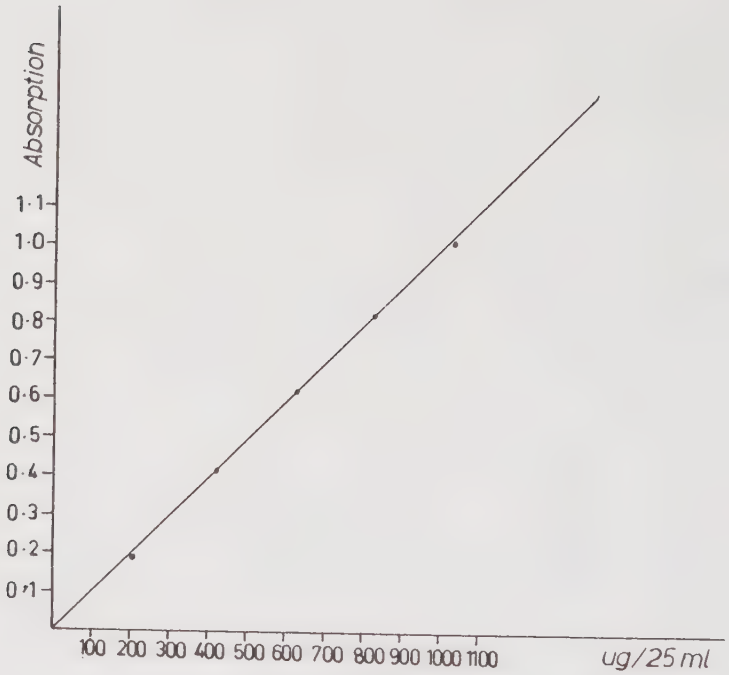


Fig. 4: Beer's law plot for the hydrazone obtained from N-methylphthalamic aldehyde in methanolic potassium hydroxide solution; data from Fig. 3 and Table 5 (λ_{max} = 612 nm).

$y = 0.000985X - 0.000572$

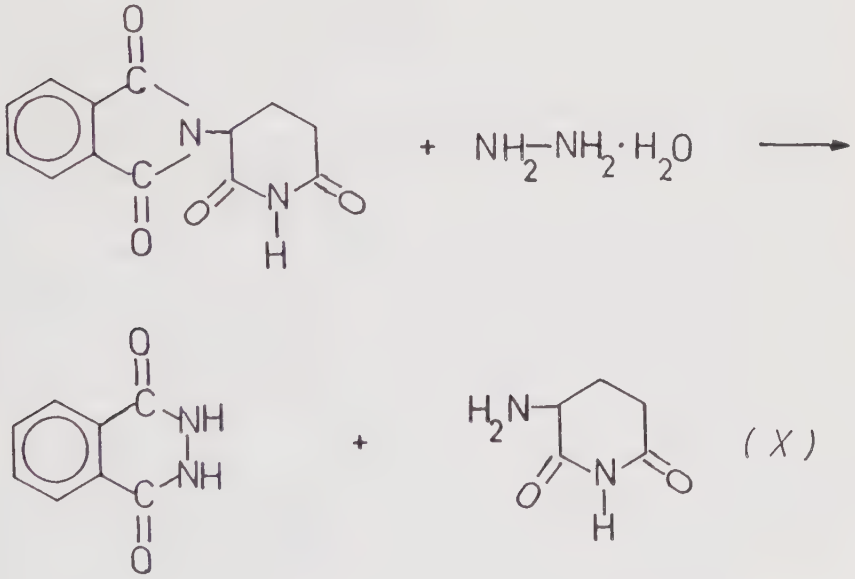
$r = 0.9993$ (correlation coefficient)

249 mg Hydrazone ≙ 200 mg Acid

The suggested method has been applied successfully to the analysis of the main hydrolysis products of thalidomide separately after their synthesis, or in mixture after the hydrolysis of thalidomide at different pH values.

3.3.3. α-(o-Carboxybenzamido)glutarimide (IV)

Reaction of D,L-α-aminoglutarimide (X) with phthalic anhydride in dry pyridine gave α-(o-carboxybenzamido)glutarimide (IV). D,L-α-aminoglutarimide was prepared by Sondheimer [61,61] through an about nine steps pathway. In this work it was prepared using lit. [63] after some modifications, this synthesis was achieved by the action of hydrazine hydrate on thalidomide itself.



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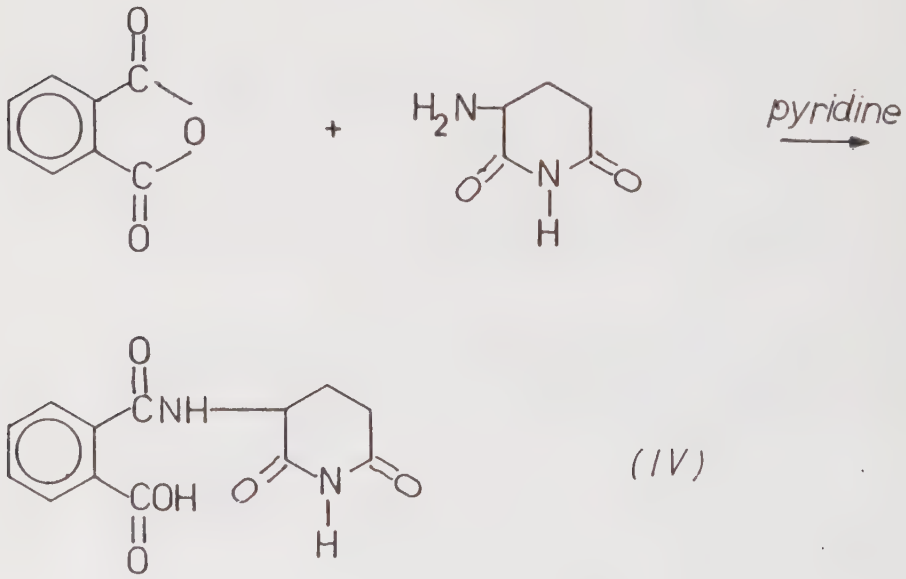
61) E.Sondheimer and R.W.Holly: J.Am.Chem.Soc. 76, 2467 (1954).

62) E.Sondheimer and R.W.Holly: ibid. 79, 3767 (1957).

63) Fr.Pat. 1.341.603 (1963); ref. C.A. 60, 6794 (1964).

Because of the instability of D,L-α-aminoglutarimide as a base it was prepared as its hydrochloride salt, which is stable. Infrared spectroscopy, ^{1}H.n.m.r. spectroscopy, mass spectroscopy and microanalysis confirm the above assigned structure of the hydrochloride salt.

The desired α-(o-carboxybenzamido)glutarimide was prepared by coupling of phthalic anhydride with D,L-aminoglutarimide after its liberation as a free base from its hydrochloride salt. The coupling was achieved in dry pyridine at room temperature [28].



(IV)

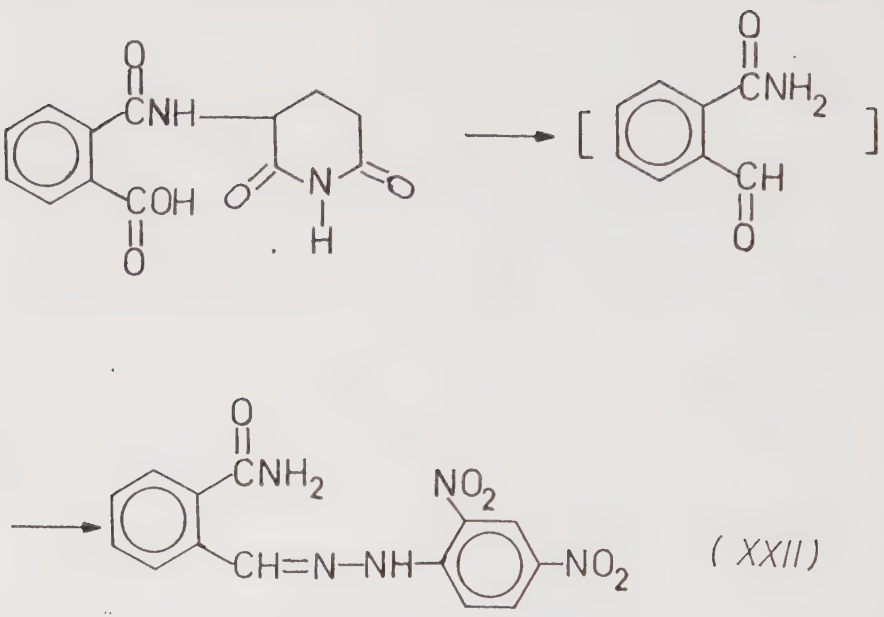
Infrared spectroscopy, ^{1}H.n.m.r. spectroscopy and microanalysis confirm the above assigned structure of α-(o-carboxybenzamido)glutarimide.

3.3.3.1. Reduction of α-(o-Carboxybenzamido)glutarimide to its Aldehyde by Use of Bis(4-methylpiperazinyl)aluminium Hydride

After the reduction of α-(o-carboxybenzamido)glutarimide by use of bis(4-methylpiperazinyl)aluminum hydride for different periods and analysing the obtained aldehyde gravimetrically with 2,4-dinitrophenylhydrazine, it was found, that the obtained 2,4-dinitrophenylhydrazone as shown in infrared spectroscopy and ^{1}H.n.m.r. spectroscopy, contained no more the glutarimide ring as a whole. It can be concluded, that after long time reduction the glutarimide ring was destroyed with the formation of the aldehyde of phthalamic acid and hence, its hydrazone on reaction with 2,4-dinitrophenylhydrazine. Moreover, when methanolic potassium hydroxide was added to the obtained hydrazone, it gave a violet and not a blue colour with absorption maximum at 582 nm.

Many attempts were done for controlling the reduction processes to avoid the destruction of the glutarimide and hence, obtaining a blue colour instead of a violet colour, but these attempts were unsuccessful and decreasing the reduction time from 12 hrs to 8, 6, 4 hrs did not pay and in all these cases the same hydrazone was obtained (XXII) in different yields.

Figure 5 shows a Beer's law study of the hydrazone, obtained from α-(o-carboxybenzamido)glutarimide after its reduction to the aldehyde.
Table 6 gives the data with which Fig. 6 was plotted.



(XXII)

Table 6

Concentration µg/25 ml	Absorption 274 nm	Absorption 428 nm	Absorption 582 nm
154	0.217	0.1226	0.1250
308	0.449	0.2460	0.2414
426	0.728	0.3950	0.3535
616	0.990	0.5350	0.5106
770	1.200	0.6660	0.6400

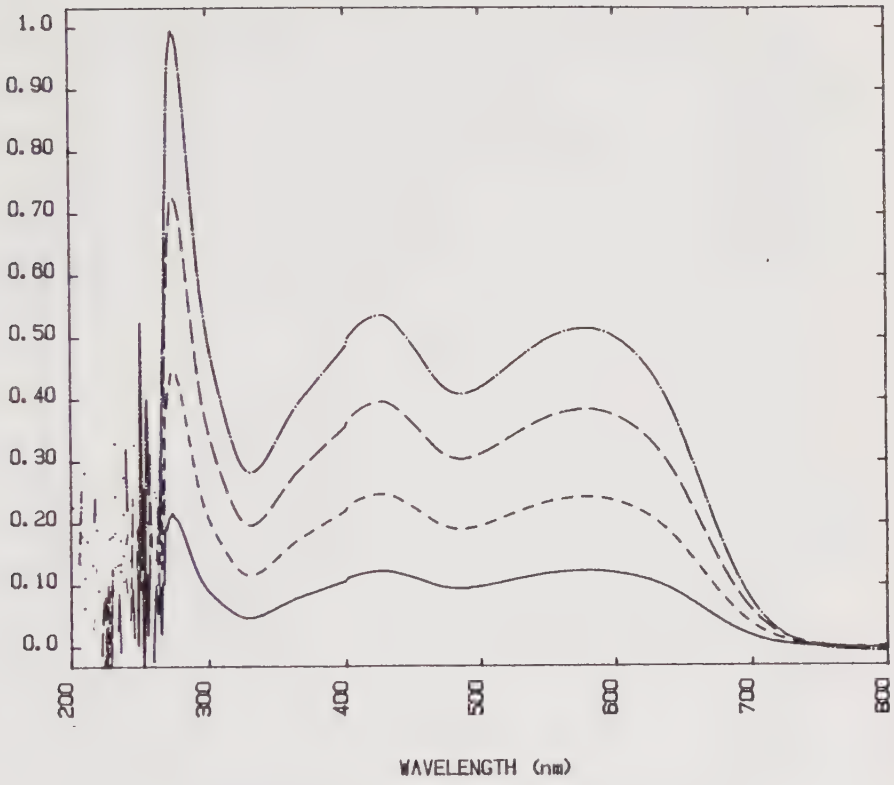


Fig. 5: Absorption spectra of four solutions of the hydrazone obtained from α-(o-carboxybenzamido)glutarimide in methanolic potassium hydroxide solution.

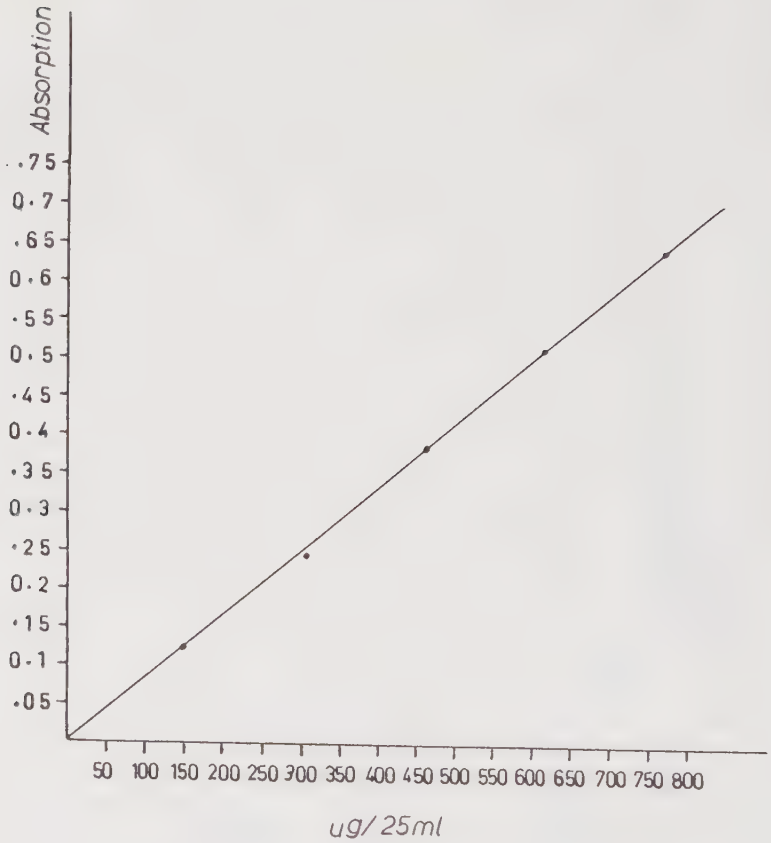
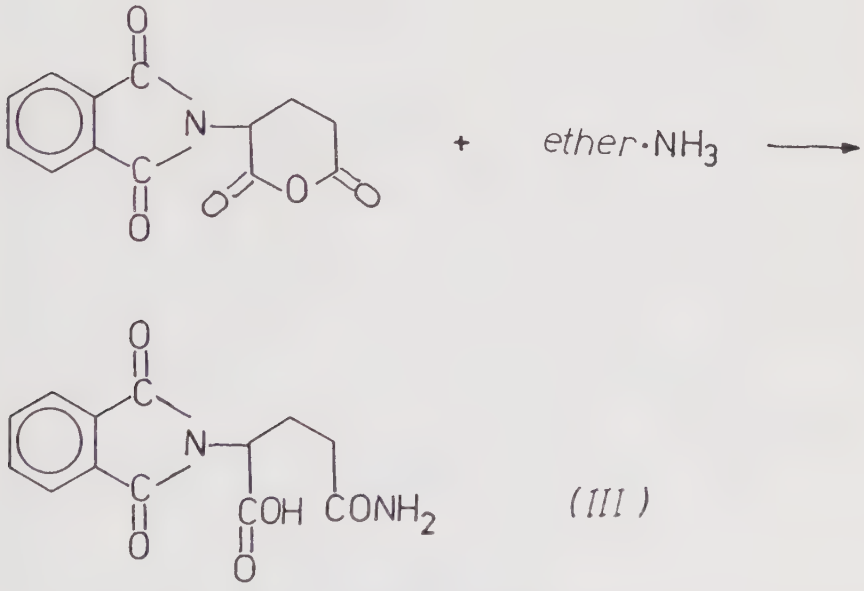


Fig. 6: Beer's law plot for the hydrazone obtained from α-(o-carboxybenzamido)glutarimide in methanolic potassium hydroxide solution; data from Fig. 5 and Table 6 (λ_{max} = 582 nm).

y = 0.0008429X - 0.01518
r = 0.9981 (correlation coefficient)
83.44 mg Hydrazone ≙ 100 mg Acid

3.3.4. 2-Phthalimidoglutaramic Acid

2-Phthalimidoglutaramic acid was prepared as a second example of thalidomide hydrolysis products in order to study the properties of the 2,4-dinitrophenylhydrazone which is obtained after the reduction of this acid to the aldehyde. It was prepared according to King et al. [64] by the action of ethereal ammonia on phthalyl-D,L-glutamic anhydride, which was prepared from L-glutamic acid and phthalic anhydride in pyridine.



(III)

Infrared spectroscopy, ^{1}H.n.m.r. spectroscopy and microanalysis confirm the above structure of 2-phthalimidoglutaramic acid.

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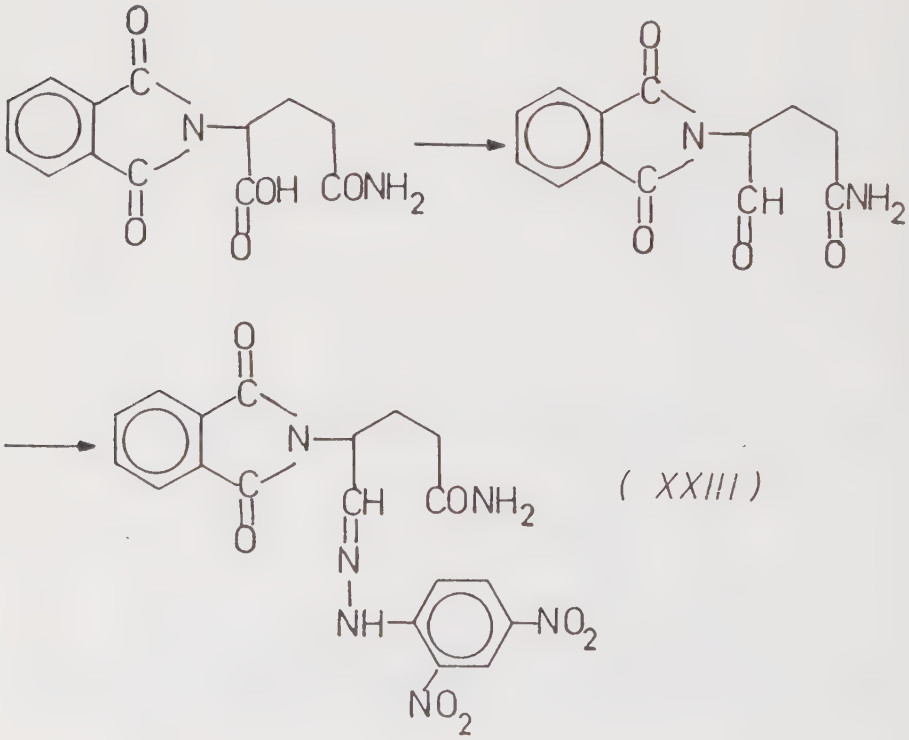
64) F.E.King and D.A.A.Kidd: J.Chem.Soc. 3315 (1949).

3.3.4.1. Reduction of 2-Phthalimidoglutaramic Acid to its Aldehyde by Use of Bis(4-methylpiperazinyl)aluminium Hydride

To maintain a similar condition for the reduction process in all cases, 2-phthalimidoglutaramic acid was reduced with bis-(4-methylpiperazinyl)aluminium hydride under reflux for 12 hrs. After the end of the reduction, the free aldehyde was obtained by hydrolysis with water. It was analysed gravimetrically with 2,4-dinitrophenylhydrazine.



(XXIII)

When methanolic potassium hydroxide solution was added to the above hydrazone, a stable blue colour with absorption maximum at 606 nm was obtained. The relation between the absorption of the blue colour at 606 nm and the concentration of the hydrazone is linear.

Table 7

Concentration µg/25 ml	Absorption at 422 nm	Absorption at 606 nm
280	0.3440	0.3470
560	0.5670	0.6330
840	0.6831	0.8357
1120	0.8990	1.1050
1400	1.0700	1.3750

Figure 7 shows a Beer's law study of the hydrazone, obtained from 2-phthalimidoglutaramic acid after its reduction to the aldehyde.
Table 7 gives the data with which Fig. 8 was plotted.

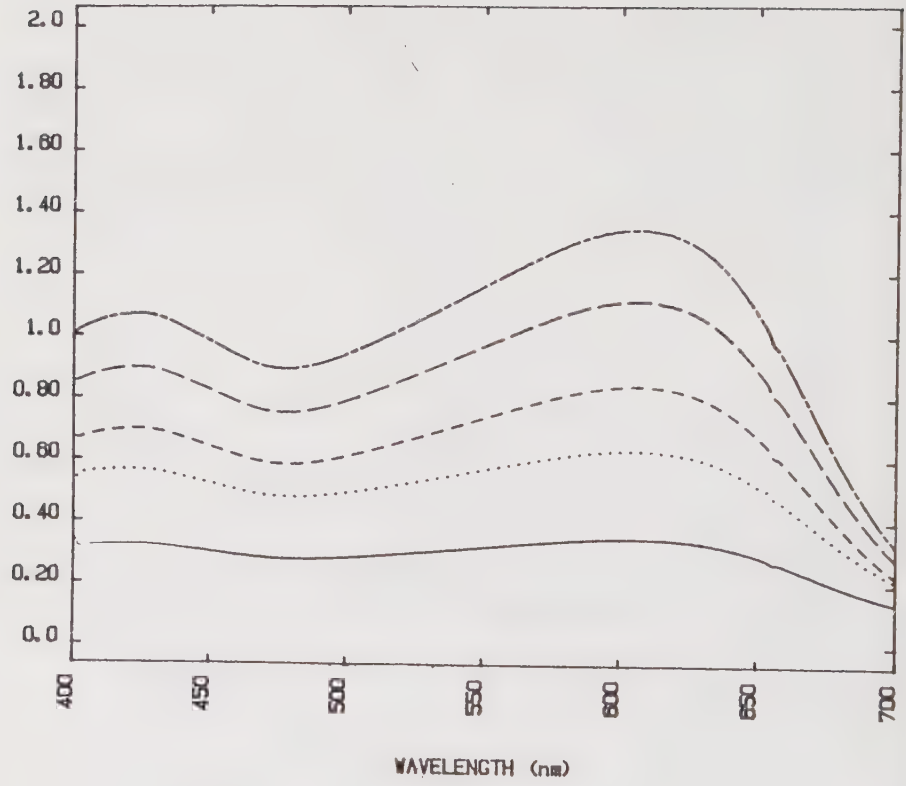


Fig. 7: Absorption spectra of five solutions of the hydrazone obtained from 2-phthalimidoglutaraldehyde in methanolic potassium hydroxide solution.

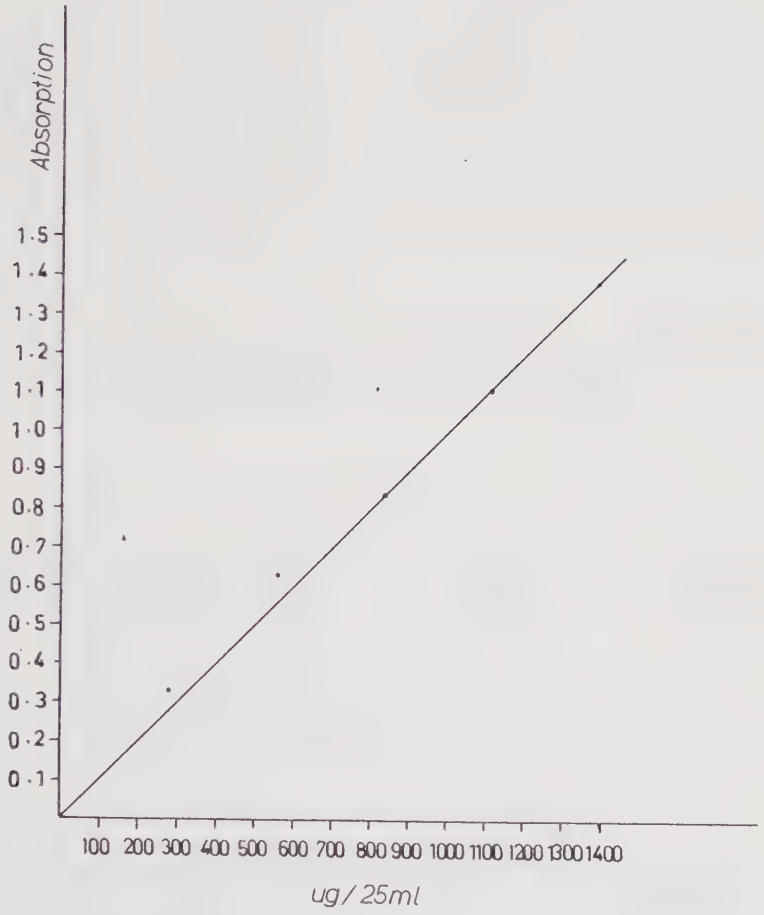


Fig. 8: Beer's law plot for the hydrazone obtained from 2-phthalimidoglutaraldehyde in methanolic potassium hydroxide solution; data from Fig. 7 and Table 7 (λ_{max} = 606 nm).

$y = 0.0009029X + 0.10074$

$r = 0.9988$

111.592 mg Hydrazone ≙ 100 mg Acid

3.3.5. 4-Phthalimidoglutaramic Acid

It was prepared according to King et al.[65] by heating phthalyl-D,L-glutamic anhydride with benzyl alcohol to give γ-benzyl hydrogen phthalyl-D,L-glutamate, which on treatment with phosphorous pentachloride and then ethereal ammonia afforded phthalyl-D,L-isoglutamine benzyl ester. From the phthalyl-D,L-isoglutamine benzyl ester 4-phthalimidoglutaramic acid was obtained by hydrogenation over palladium-charcoal.

3.3.5.1. Reduction of 4-Phthalimidoglutaramic Acid to its Aldehyde by Use of Bis(4-methylpiperazinyl)aluminium Hydride

Before the reduction of 4-phthalimidoglutaramic acid was carried out, it was dried at 100°C under vacuum in order to lose any water of crystallization. The loss of all water of crystallization was confirmed by the microanalysis of the dried compound.

The dried compound was reduced by the use of bis(4-methylpiperazinyl)aluminium hydride under reflux for 12 hrs and the resulting aldehyde was analysed gravimetrically with 2,4-dinitrophenylhydrazine.

When alcoholic potassium hydroxide solution was added to the obtained hydrazone, a stable blue colour with absorption maximum at 614 nm was obtained. The relation between the absorption of the blue colour at 614 nm and the concentration of the hydrazone is linear.

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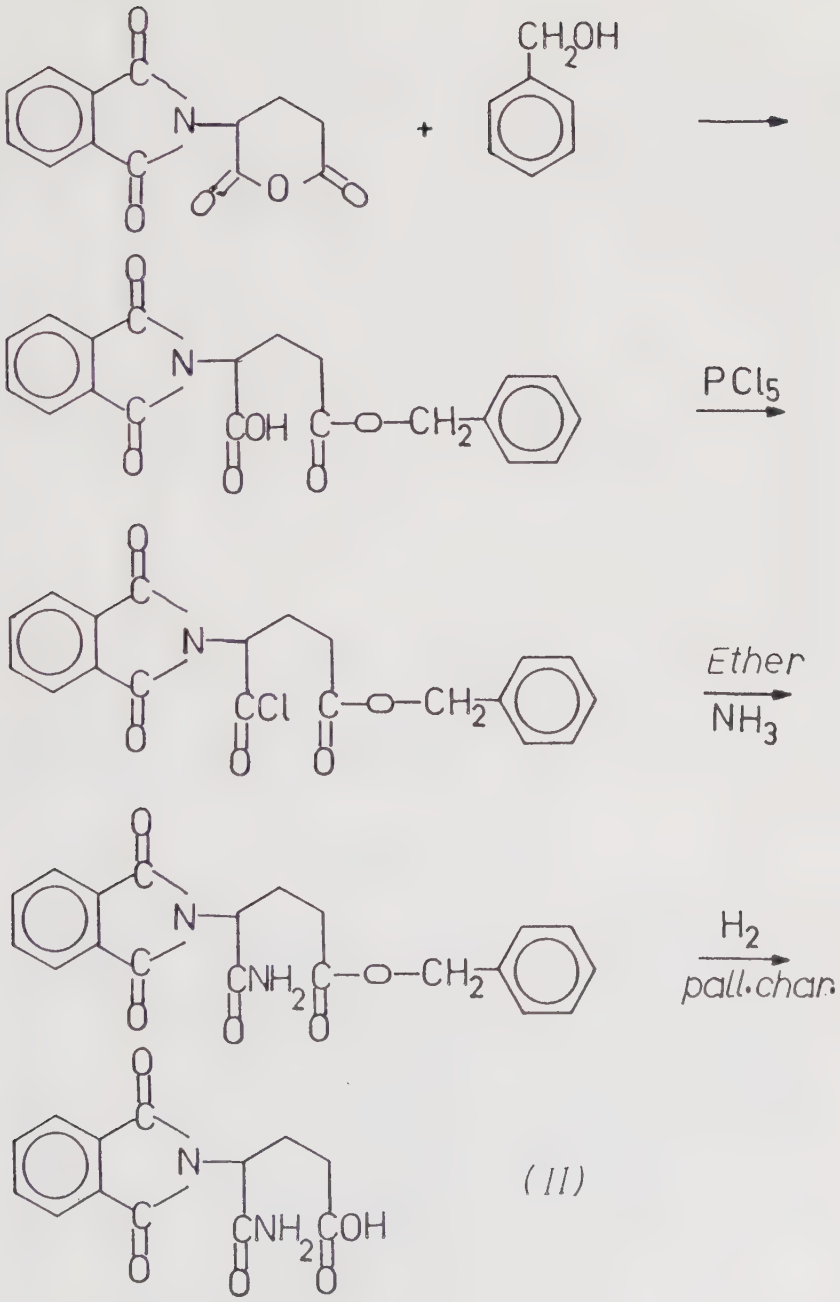
65) F.E.King, B.S.Jackson and D.A.A.Kidd: J.Chem.Soc. 243 (1951).



(II)

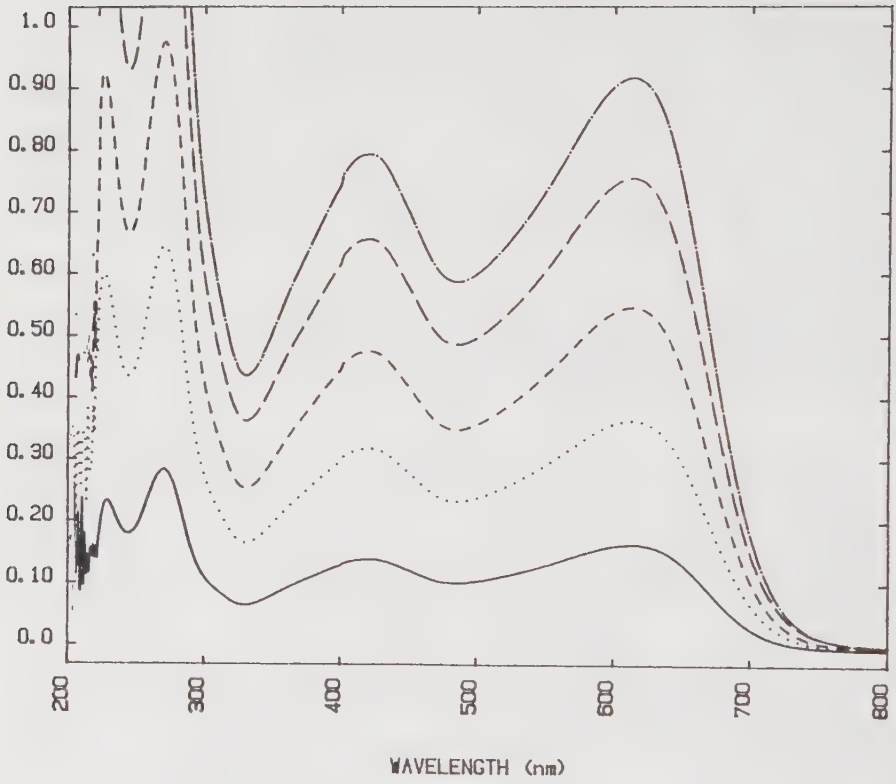
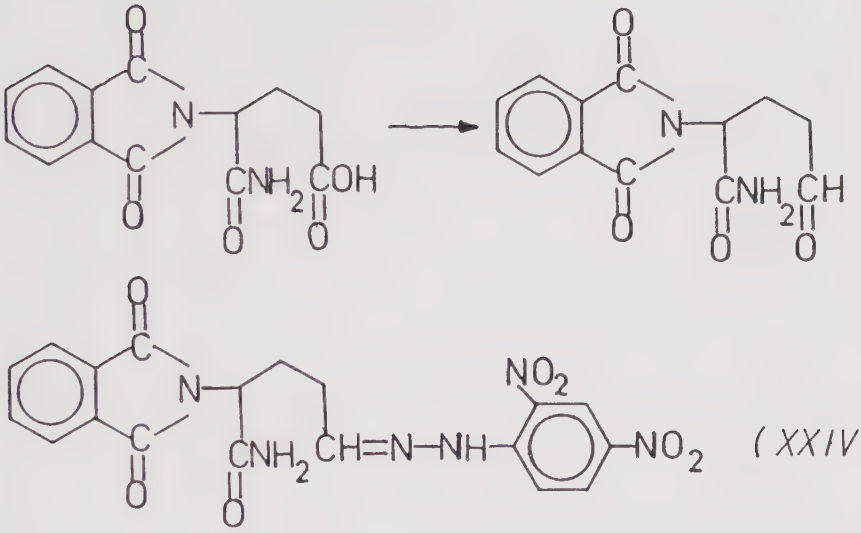


Fig. 9: Absorption spectra of five solutions of the hydrazone obtained from 4-phthalimidoglutaraldehyde in methanolic potassium hydroxide solution.



(XXIV)

Figure 9 shows a Beer's law study of the hydrazone obtained from 4-phthalimidoglutaramic acid after its reduction to the aldehyde.
Table 8 gives the data with which Fig. 10 was plotted.

Table 8

Concentration µg/25 ml	Absorption at 229 nm	Absorption at 271 nm	Absorption at 420 nm	Absorption at 614 nm
160	0.233	0.2830	0.1387	0.1638
320	0.601	0.6451	0.3179	0.3565
480	0.924	0.9750	0.4740	0.5565
640	1.295	1.3430	0.6559	0.7558
800	1.585	1.6260	0.7933	0.9250

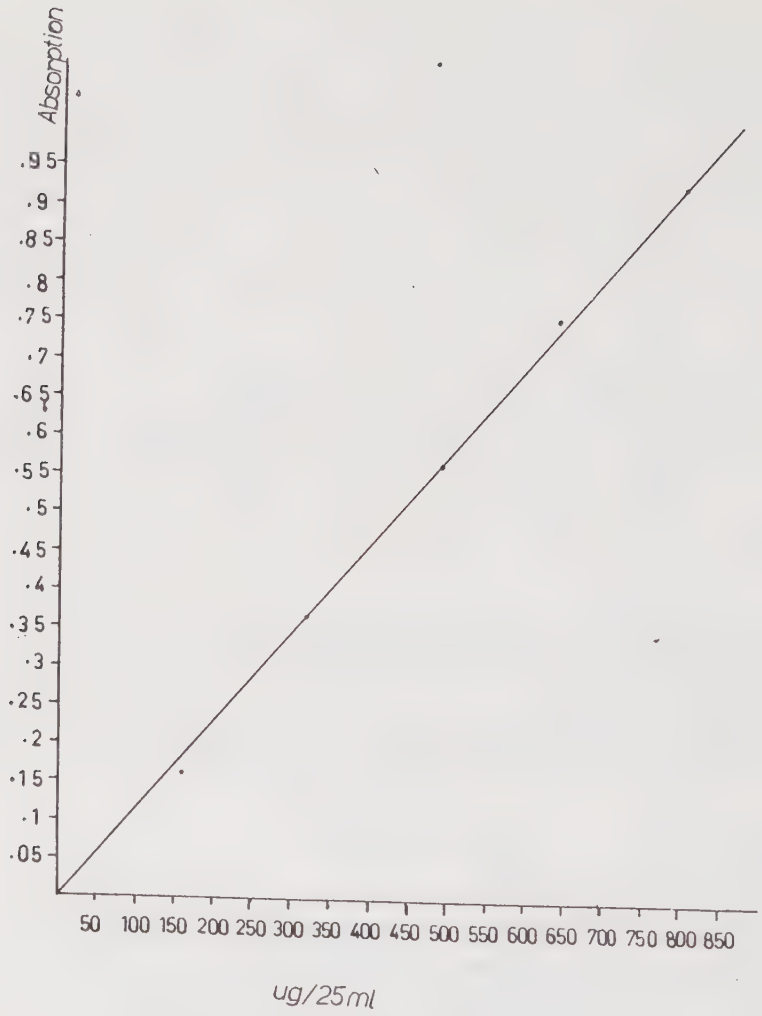


Fig. 10: Beer's law plot for the hydrazone obtained from 4-phthalimidoglutaraldehyde in methanolic potassium hydroxide solution; data from Fig. 9 and Table 8 (λ_{max} = 614 nm).

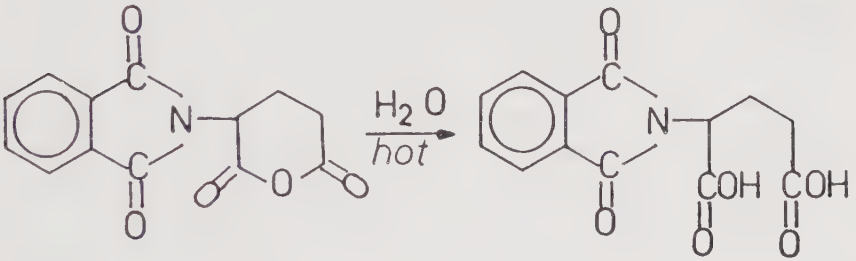
y = 0.00120X - 0.02499

r = 0.9995

116.377 mg Hydrazone ≙ 100 mg Acid

3.3.6. 2-Phthalimidoglutaric Acid

It was prepared by hydrolysis of phthalyl-D,L-glutamic anhydride with boiling water [64]. Infrared spectroscopy, ^{1}H.n.m.r. spectroscopy and microanalysis confirm the structure of 2-phthalimidoglutaric acid.



(V)

3.3.6.1. Reduction of 2-Phthalimidoglutaric Acid to its Aldehyde by Use of Bis(4-methylpiperazinyl) aluminium Hydride

For the reduction of 2-phthalimidoglutaric acid, the amount of the reagent used was doubled, because of the presence of two carboxylic groups. It was reduced under reflux for 12 hrs and the resulting aldehyde was analysed gravimetrically with 2,4-dinitrophenylhydrazine.

When alcoholic potassium hydroxide solution was added to the obtained hydrazone, a stable blue colour with absorption maximum at 604 nm was obtained. The relation between the absorption of the blue colour at 604 nm and the concentration of the hydrazone is linear.

Figure 11 shows a Beer's law study of the hydrazone, obtained from 2-phthalimidoglutaric acid after its reduction to the aldehyde.
Table 9 gives the data with which Fig. 12 was plotted.

Table 9

Concentration µg/25 ml	Absorption at 426 nm	Absorption at 604 nm
240	0.257	0.281
480	0.467	0.528
720	0.733	0.830
960	0.977	1.093

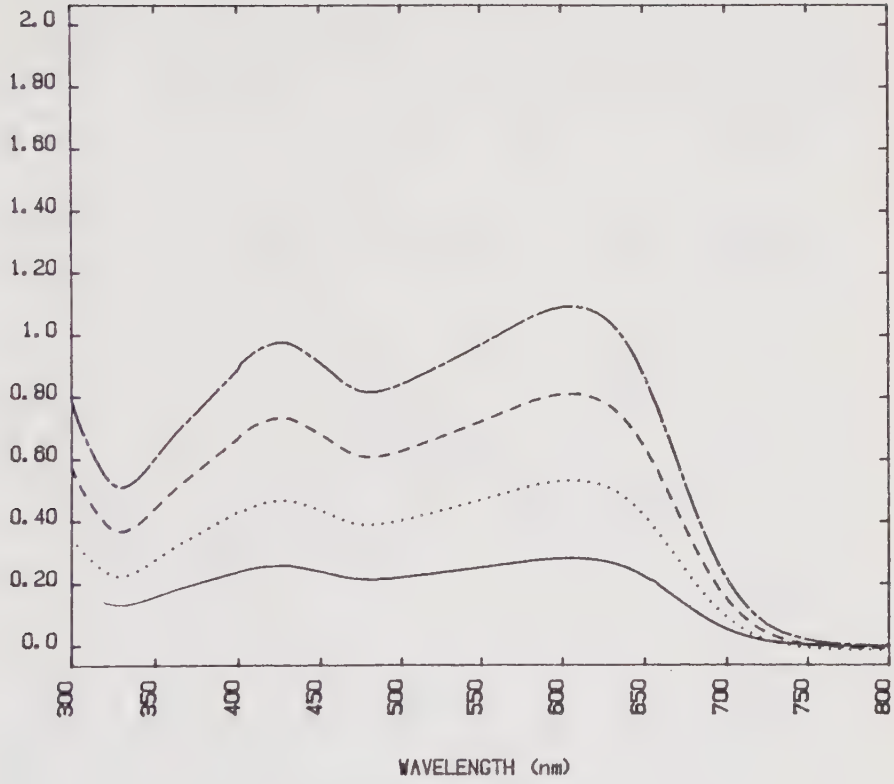


Fig. 11: Absorption spectra of four solutions of the hydrazone obtained from 2-phthalimidoglutaraldehdye in methanolic potassium hydroxide solution.

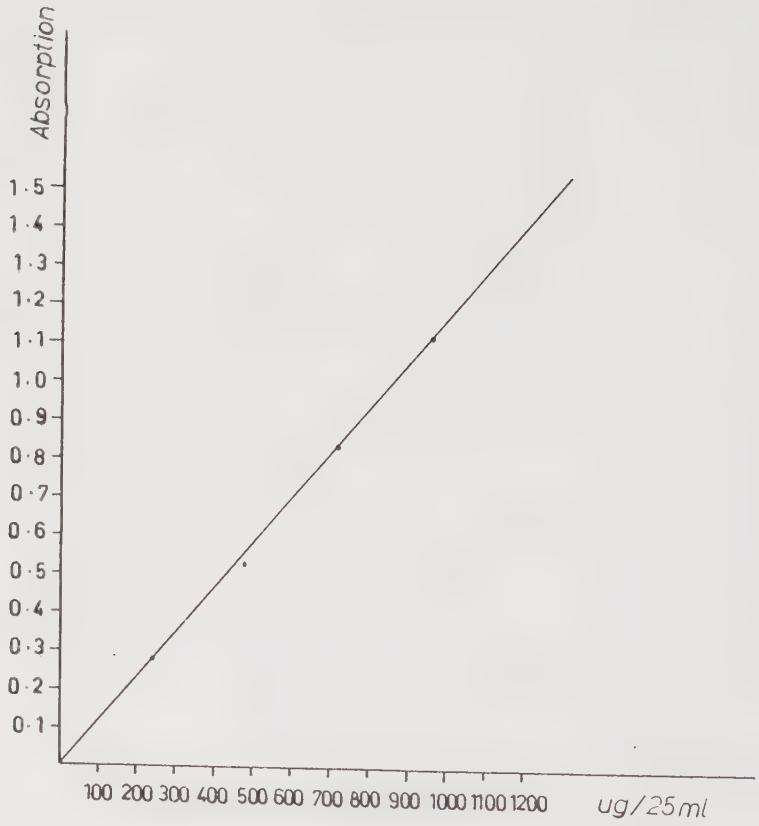
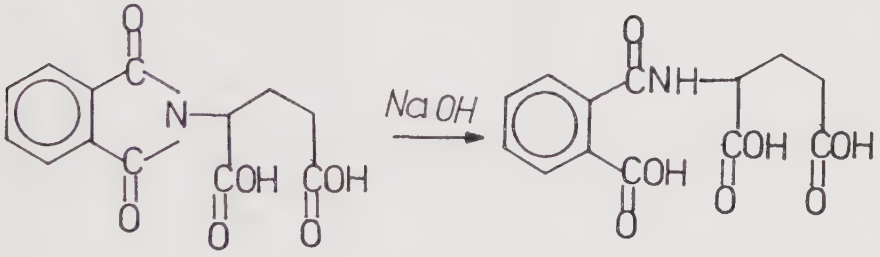


Fig. 12: Beer's law plot for the hydrazone obtained from 2-phthalimidoglutaraldehyde in methanolic potassium hydroxide solution; data from Fig. 11 and Table 9 (λ_{max} = 604 nm).
y = 0.0011408X - 0.0015
r = 0.9993
128.86 mg Hydrazone ≙ 100 mg Acid

3.3.7. 2-(o-Carboxybenzamido)glutaric Acid

2-Phthalimidoglutaric acid was hydrolysed with 2N sodium hydroxide to afford 2-(o-carboxybenzamido)glutaric acid. The alkaline solution was neutralized with formic acid, then the neutral solution was filtered over an ion exchanger (Amberlite IR 120, H^+ form) column and the eluent was dried in vacuum 28).

Infrared spectroscopy, ^{1}H.n.m.r. spectroscopy and microanalysis confirm the structure of 2-(o-carboxybenzamido)glutaric acid.



(VIII)

3.3.7.1. Reduction of 2-(o-Carboxybenzamido)glutaric Acid by Use of Bis(4-methylpiperazinyl)aluminium Hydride

For the reduction of 2-(o-carboxybenzamido)glutaric acid, the amount of the reagent used was tripled because of the presence of three carboxylic groups. It was reduced under reflux for 12 hrs, and the resulting aldehyde was analysed gravimetrically with 2,4-dinitrophenylhydrazine.

When alcoholic potassium hydroxide solution was added to the obtained hydrazone, a stable blue colour with absorption maximum at 618 nm was obtained. The relation between the absorption of the blue colour at 618 nm and the concentration of the hydrazone is linear.

Figure 13 shows a Beer's law study of the hydrazone, obtained from 2-(o-carboxybenzamido)glutaric acid after its reduction to the aldehyde.
Table 10 gives the data with which Fig. 14 was plotted.

Table 10

Concentration µg/25 ml	Absorption at 428 nm	Absorption at 618 nm
194	0.218	0.263
388	0.439	0.528
582	0.687	0.748
776	0.900	1.080
970	1.160	1.376

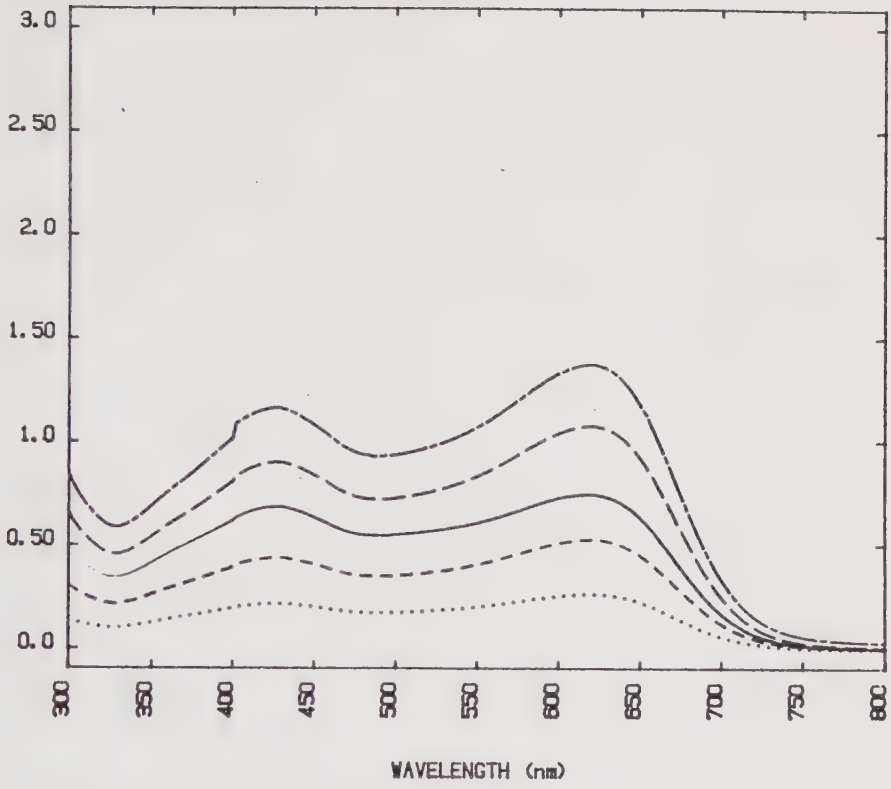


Fig. 13: Absorption spectra of five solutions of the hydrazone obtained from 2-(o-carboxybenzamido)glutaraldehyde in methanolic potassium hydroxide solution.

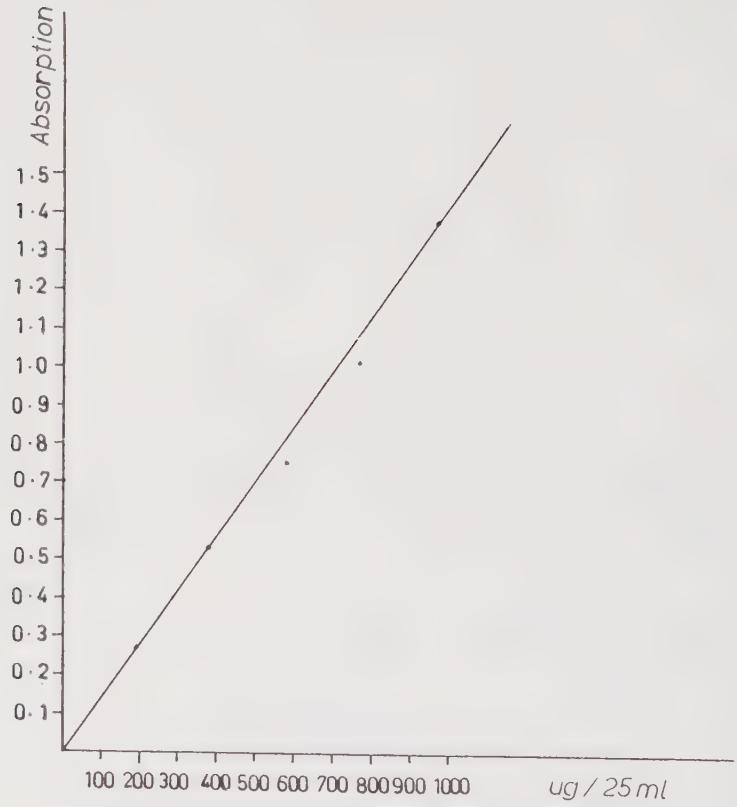


Fig. 14: Beer's law plot for the hydrazone obtained from 2-(carboxybenzamido)glutaraldehyde in methanolic potassium hydroxide solution; data from Fig. 13 and Table 10 (λ_{max} = 618 nm).

$y = 0.00143X - 0.0344$

$r = 0.9977$

146.73 mg Hydrazone ≙ 100 mg Acid

3.3.8. Quantitative Colour Reaction of the Mixture of Thalidomide Hydrolysis Products

Informations obtained from the visible absorption spectra of colour were completed by the application of the previous suggested method on thalidomide itself. It could be concluded that, when a mixture of thalidomide hydrolysis products was reduced by use of the same method and the obtained aldehydes mixture was analysed gravimetrically with 2,4-dinitrophenylhydrazine, then alcoholic potassium hydroxide solution was added to the obtained mixture of 2,4-dinitrophenylhydrazones: The absorption band of the obtained colour was due to overlapping bands, which are frequently seen in the ultraviolet and visible absorption spectra of molecules. The extinction coefficients of these bands are often taken to be the measured values. This is not really correct, although it would suffice for most qualitative and quantitative work. Since the measured absorbance values will actually have contributions from more than one band due to the overlapping in the regions 420-428 nm and 604-618 nm, and this is true because after the suggested method was applied on the main hydrolysis products of thalidomide separately, the colour which was obtained in each case had an absorption maximum at 604-618 nm, except in case of α-(o-carboxybenzamido)glutarimide, which due to the destruction of the glutarimide ring during the reduction under reflux, the violet colour obtained had an absorption maximum at 582 nm, compared with N-methylphthalamic acid, the blue colour obtained had absorption maximum at 612 nm.

The suggested method had been applied successfully for the analysis of small quantities of thalidomide via the mixture of its hydrolysis products. When a certain quantity of thalidomide was hydrolysed at different pH values and the mixture of the hydrolysis products was reduced by use of bis(4-methylpiperazinyl)aluminium hydride as a selective reducing reagent of carboxylic acids to aldehydes, and the obtained mixture of aldehydes was analysed gravimetrically with 2,4-dinitrophenyl-

hydrazine, the amount of the resulting hydrazones can be related to the amount of thalidomide previously hydrolysed according to the value of absorption of colour, which developed when alcoholic potassium hydroxide was added to the mixture of hydrazones obtained from thalidomide.

A calibration curve relates the absorption maximum of the colour produced and the concentration of the hydrazones (µg/25 ml), and hence the amount of thalidomide hydrolyzed; any unknown quantity of thalidomide can be measured after applying the same steps for the development of the colour.

The suggested method was at first applied on a mixture of thalidomide hydrolysis products which were prepared previously. The quantity of each hydrolysis product in this mixture was according to the percentage amounts which were obtained by Beckmann [7] and listed in Table 1, p. 15.

A few milligrams of the mixture of hydrolysis products was used in order to examine the efficiency of the suggested method for the quantitative analysis of thalidomide. The mixture was reduced under reflux for 12 hrs and the resulting mixture of aldehydes was analysed gravimetrically with 2,4-dinitrophenylhydrazine.

When alcoholic potassium hydroxide was added to the obtained hydrazones, a purple colour was produced at first and after standing one hour it changed to a stable blue colour with absorption maximum at 616 nm. The relation between the absorption of the blue colour at 616 nm and the concentration of the hydrazones (µg/25 ml) is linear.

Figure 15 shows a Beer's law study of the hydrazones, obtained from the mixture of hydrolysis products after the reduction to aldehydes.
Table 11 gives the data with which Fig. 16 was plotted.

Table 11

Concentration µg/25 ml	Absorption at 430 nm	Absorption at 616 nm
120	0.1360	0.1520
240	0.2695	0.3200
360	0.4260	0.5054
480	0.5820	0.6960
600	0.7470	0.8520

The purple colour which was produced at first when alcoholic potassium hydroxide was added, may be due to the presence of phthalic aldehyde hydrazone in the mixture, while the phthalaldehyde hydrazone alone gives by addition of alcoholic potassium hydroxide a purple colour with absorption maximum at 510 nm. And also presumably because of some destruction of the glutarimide ring in α-(o-carboxybenzamido)glutarimide.

The correlation of colour and wavelength of visible light is shown in Table 12.

Table 12

Wavelength	Colour	Complement
400-450	violet	yellow-green
450-480	blue	yellow
480-490	green-blue	orange
490-500	blue-green	red
500-560	green	purple
560-575	yellow-green	violet
575-590	yellow	blue
590-625	orange	green-blue
625-750	red	blue-green

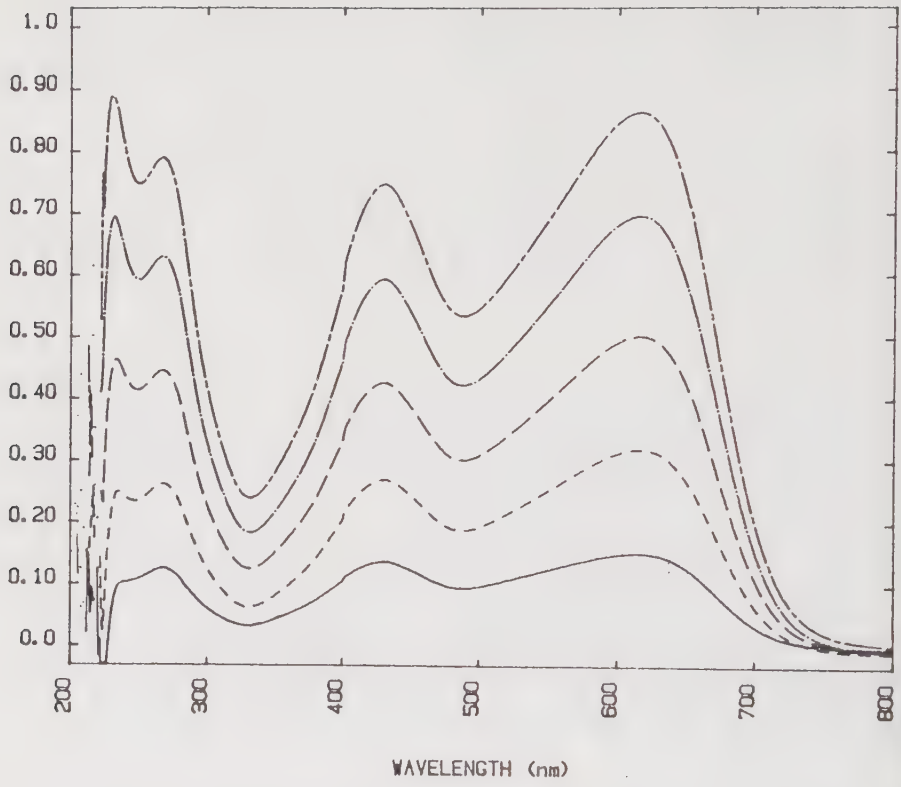


Fig. 15: Absorption spectra of five solutions of the hydrazones obtained from a mixture of thalidomide hydrolysis products (the percentage amounts obtained by Beckmann in urine of man; Tab. 1) in methanolic potassium hydroxide solution.

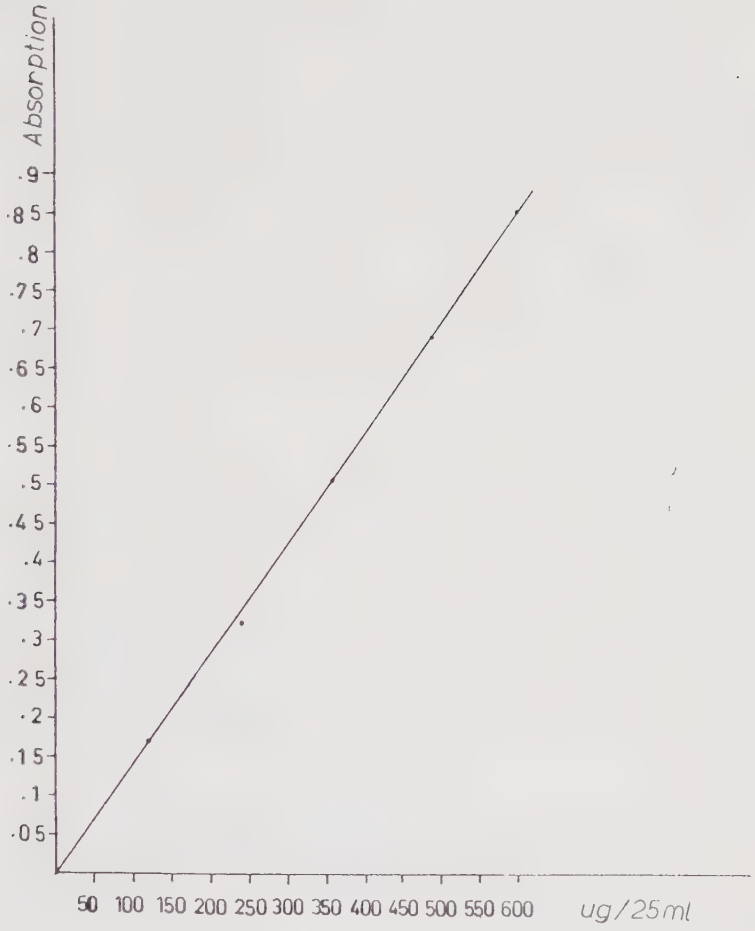


Fig. 16: Beer's law plot for the hydrazones obtained from a mixture of thalidomide hydrolysis products (as obtained by Beckmann in Tab. 1) in methanolic potassium hydroxide solution; data from Fig. 15 and Table 11 (λ_{max} = 616 nm).
y = 0.00148X - 0.02772
r = 0.9995
48 mg Hydrazones ≙ 35 mg Mixture

3.3.9. Quantitative Colour Reaction of Thalidomide, Hydrolysed at pH 7.4 and 37°C for 24 Hours

A few milligrams of thalidomide were dissolved in dioxan and phosphate buffer [66] pH 7.4 was added. The mixture was left in a water bath at 37°C for 24 hrs. After the end of the hydrolysis, the solvent was evaporated in vacuo and the residue was dried at 110°C in vacuum.
The well dried mixture of hydrolysis products was reduced by use of bis(4-methylpiperazinyl)aluminium hydride with stirring at room temperature for two days. The resulting mixture of aldehydes was analysed gravimetrically with 2,4-dinitrophenylhydrazine.

When alcoholic potassium hydroxide was added to the obtained hydrazones, a purple colour was produced at first and after standing one hour, it changed to a stable blue colour with absorption maximum at 622 nm. The blue colour was stable for several days. The relation between the absorption of the blue colour at 622 nm and the concentration of the hydrazones (µg/25 ml) is linear. The amount of the hydrazones was related to the amount of thalidomide which was previously hydrolysed.

The purple colour, which was produced at first when alcoholic potassium hydroxide was added, presumably because of some hydrolysis of α-(o-carboxybenzamido)glutarimide (IV) to phthalic acid and as mentioned, the hydrazone of phthalic aldehyde gives a purple colour.

Figure 17 shows a Beer's law study of the hydrazones, obtained after the hydrolysis of thalidomide at pH 7.4 and 37°C for 24 hrs and reduction of the hydrolysis products with stirring at room temperature for two days.
Table 13 gives the data with which Fig. 18 was plotted.

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66) R.C.Weast and S.M.Selby: "Hand Book of Chemistry and Physics" 48th ed., CRC Press, Inc. Cleveland, D 79 (1967-1968).

Table 13

Concentration µg/25 ml	Absorption at 428 nm	Absorption at 622 nm
160	0.0790	0.1170
320	0.1953	0.2712
480	0.3060	0.4410
640	0.4260	0.5820
800	0.5400	0.7300

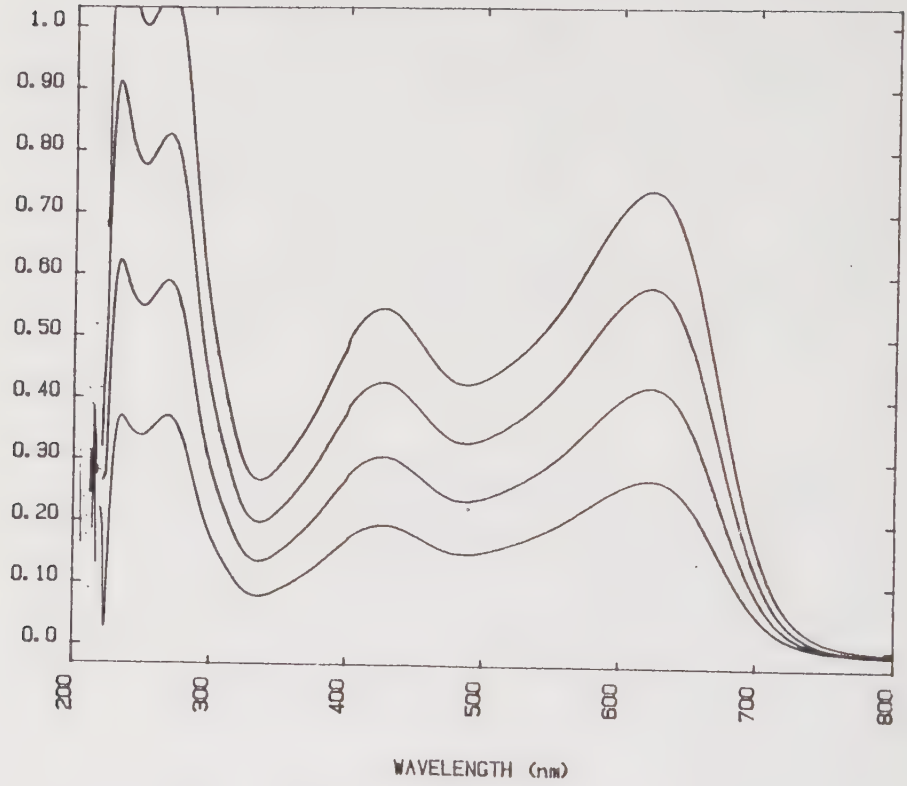

Fig. 17: Absorption spectra of four solutions of the hydrazones obtained from thalidomide (after its hydrolysis at pH 7.4, 37°C, 24 hrs and reduced in the cold) in methanolic potassium hydroxide solution.

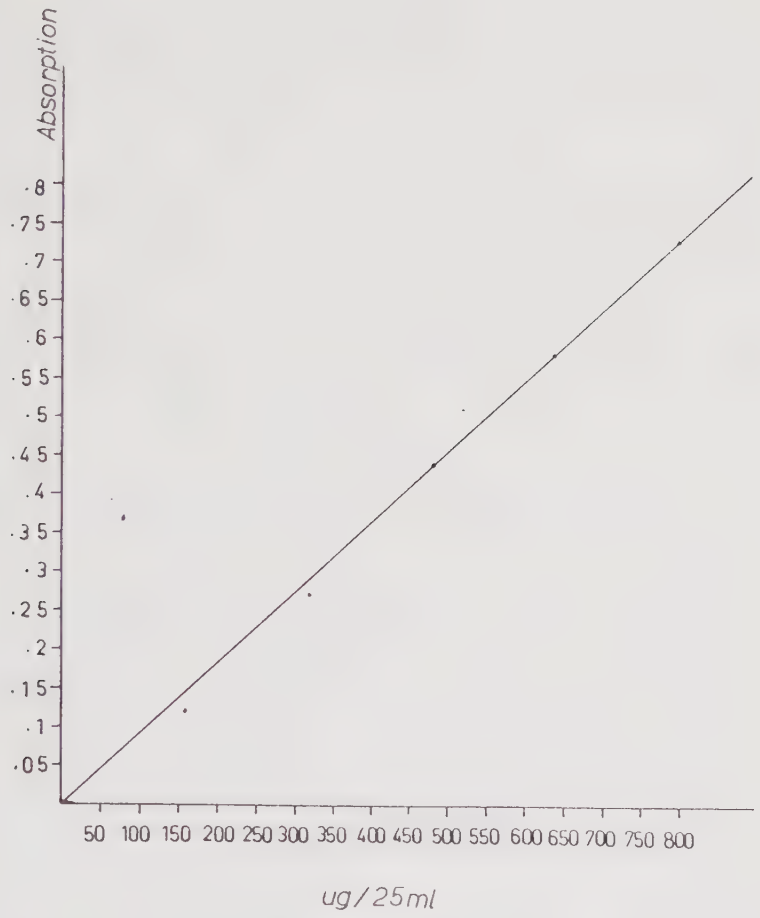

Fig. 18: Beer's law plot for the hydrazones obtained from thalidomide (hydrolysed at pH 7.4, 37°C, 24 hrs and reduced in the cold) in methanolic potassium hydroxide solution; data from Fig. 17 and Table 13 (λ_{max} = 622 nm).
y = 0.00096X - 0.0328
r = 0.9995
40 mg Hydrazones ≙ 49 mg Thalidomide

The foregoing hydrolysis steps of thalidomide at pH 7.4 and 37°C for 24 hrs were repeated and the obtained dried hydrolysis products were reduced by use of bis(4-methylpiperazinyl)aluminium hydride under reflux for 12 hrs instead of their reduction with stirring at room temperature for two days.

It was found, that when alcoholic potassium hydroxide solution was added to the obtained hydrazones, a purple colour was produced, and this purple colour remained for a longer time than in case of the cold reduction, before it changed to a stable blue colour with absorption maximum at 582 nm. This hypsochromic shift may be due to more destruction of the glutarimide ring in α-(o-carboxybenzamido)glutarimide, beside the phthalic acid, which was produced at the hydrolysis pH conditions. The relation between the absorption of the blue colour at 582 nm and the concentration of the hydrazones (µg/25 ml) is linear and does not pass through the origin.

Figure 19 shows a Beer's law study of the hydrazones, obtained after the hydrolysis of thalidomide at pH 7.4 and 37°C for 24 hrs, and reduction of the hydrolysis products under reflux for 12 hrs.
Table 14 gives the data with which Fig. 20 was plotted.

Table 14

Concentration µg/25 ml	Absorption at 432 nm	Absorption at 582 nm
160	0.1009	0.138
320	0.2380	0.307
480	0.3870	0.489
640	0.5130	0.665
800	0.6750	0.847

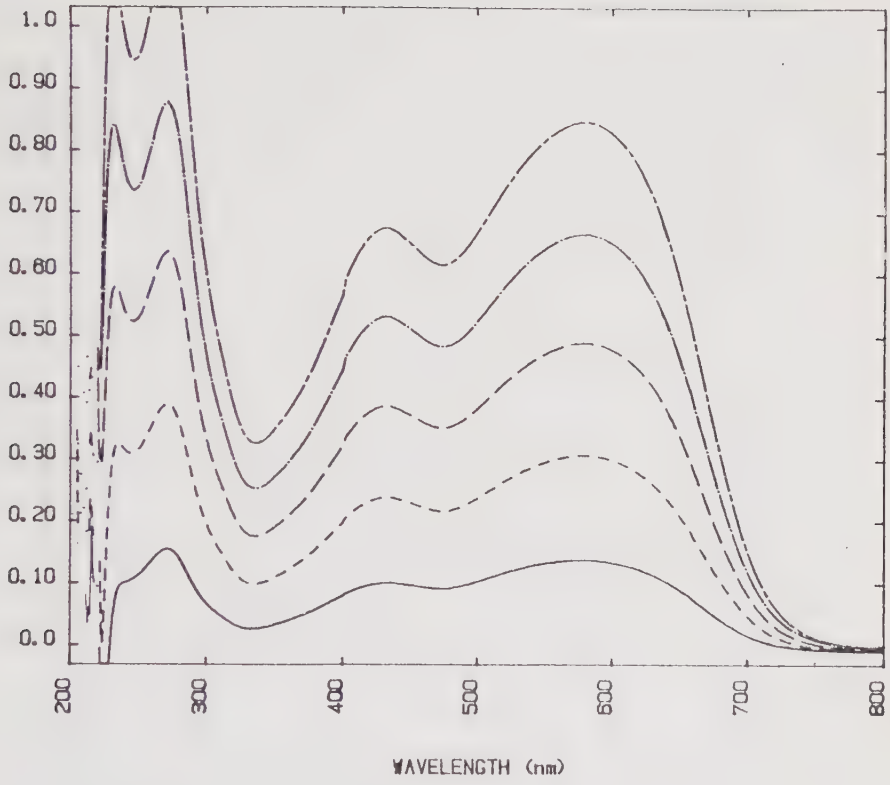

Fig. 19: Absorption spectra of five solutions of the hydrazones obtained from thalidomide (after its hydrolysis at pH 7.4, 37°C, 24 hrs, and reduction under reflux) in methanolic potassium hydroxide solution.

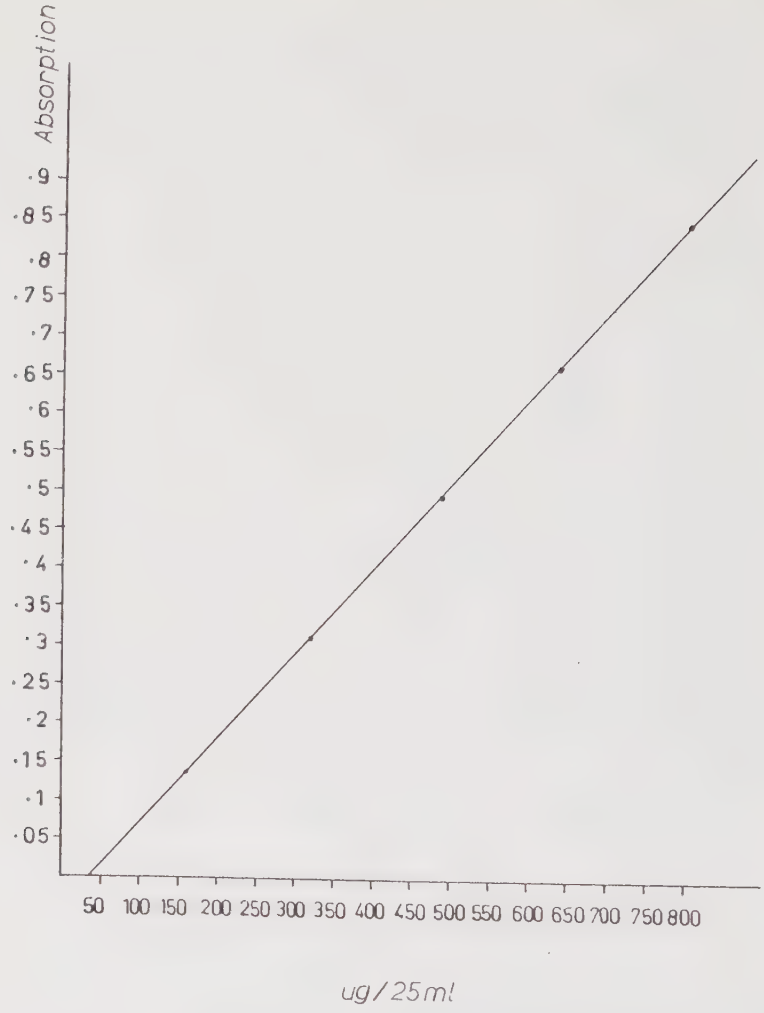

Fig. 20: Beer's law plot for the hydrazones obtained from thalidomide (hydrolysed at pH 7.4, 37°C, 24 hrs and reduced under reflux) in methanolic potassium hydroxide solution; data from Fig. 19 and Table 14 (λ_{max} = 582 nm).
y = 0.00111X - 0.0436
r = 0.9999
80 mg Hydrazones ≙ 53.5 mg Thalidomide

3.3.10. Quantitative Colour Reaction of Thalidomide, Hydrolysed at pH 7.4 and 37°C for 4 Hours

A few milligrams of thalidomide were dissolved in dioxan and phosphate buffer pH 7.4 was added. The mixture was left in a water bath at 37°C for 4 hrs. After the end of the hydrolysis the solvent was evaporated in vacuo and the residue was dried at 110°C in vacuum.
The well dried hydrolysis products were reduced by use of bis(4-methylpiperazinyl)aluminium hydride under reflux for 12 hrs. The resulting mixture of aldehydes was analysed gravimetrically with 2,4-dinitrophenylhydrazine.
When alcoholic potassium hydroxide was added to the obtained hydrazones, a purple colour was produced at first and this colour remained for a short time, then it changed to stable blue colour with absorption maximum at 622 nm, when it was measured as usual after one hour. The relation between the absorption of the blue colour at 622 nm and the concentration of the hydrazones (µg/25 ml) is linear. The amount of the hydrazones was related to the amount of the thalidomide which was previously hydrolysed.

It can be concluded, that at physiological pH 7.4, thalidomide is rapidly hydrolysed. At this pH large amounts of secondary hydrolysis products appeared, particularly phthalic acid, 2- and 4-(o-carboxybenzamido)glutaramic acid and the amount of the secondary hydrolysis products particularly phthalic acid was dependent on the time of the hydrolysis of thalidomide. At this pH the glutarimide ring is susceptible to hydrolysis to give 2- and 4-phthalimidoglutaramic acid (II & III). However, compound (II) is very stable at pH 7.4.

Figure 21 shows a Beer's law study of the hydrazones, obtained after the hydrolysis of thalidomide at pH 7.4 and 37°C for 4 hrs, and reduction of the hydrolysis products under reflux 12 hrs.
Table 15 gives the data with which Fig. 22 was plotted.

Table 15

Concentration µg/25 ml	Absorption at 229 nm	Absorption at 271 nm	Absorption at 426 nm	Absorption at 622 nm
192.5	0.2697		0.2910	0.291
385.0	0.6627	0.6047	0.3284	0.634
577.5	1.0360	0.9260	0.4930	0.952
770.0	1.4200	1.2340	0.6499	1.267
962.5	1.7800	1.5430	0.8020	1.586

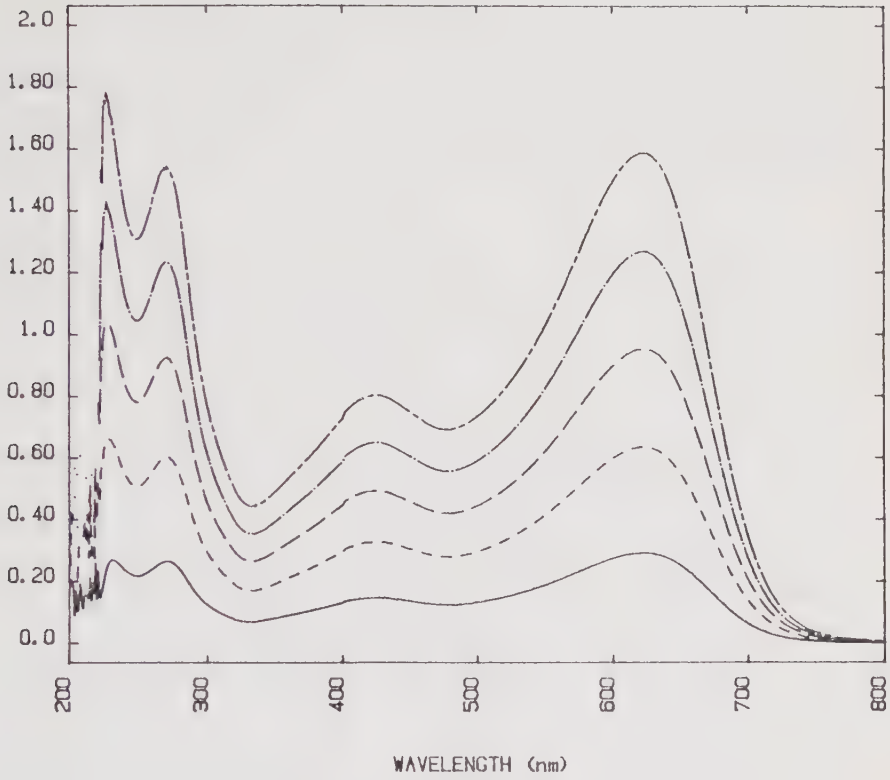

Fig. 21: Absorption spectra of five solutions of the hydrazones obtained from thalidomide (after its hydrolysis at pH 7.4, 37°C, 4 hrs and reduction under reflux) in methanolic potassium hydroxide solution.

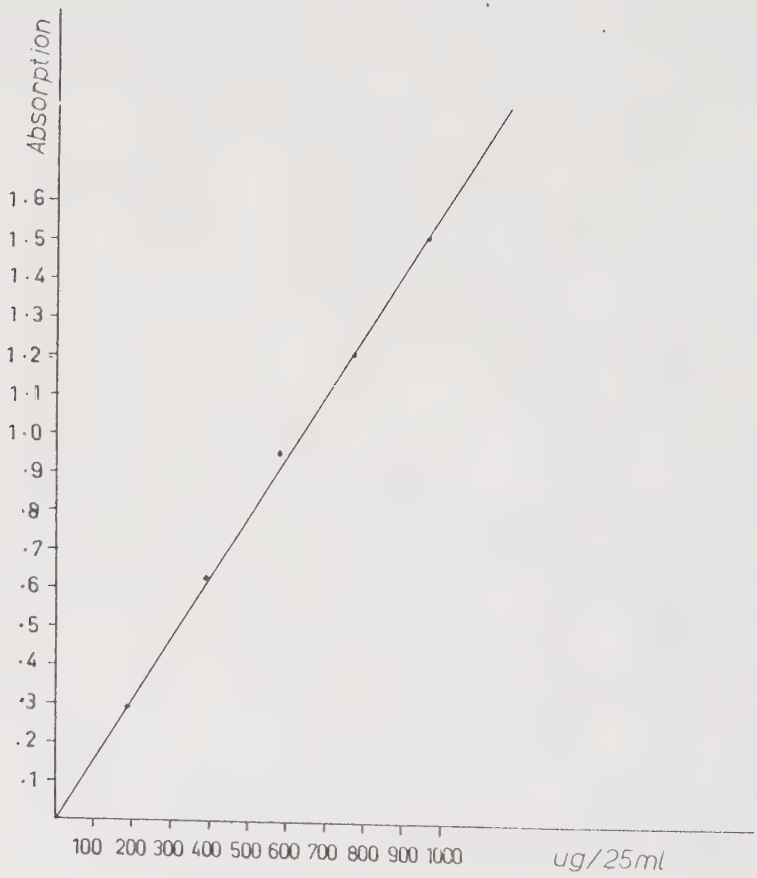

Fig. 22: Beer's law plot for the hydrazones obtained from thalidomide (hydrolysed at pH 7.4, 37°C, 4 hrs and reduced under reflux) in methanolic potassium hydroxide solution; data from Fig. 21 and Table 15 (λ_{max} = 622 nm).

$y = 0.00167X - 0.0209$

$r = 0.9998$

38.5 mg Hydrazones ≙ 43 mg Thalidomide

3.3.11. Quantitative Colour Reaction of Thalidomide, Hydrolysed at pH 8 and 37°C for 24 Hours

A few milligrams of thalidomide were dissolved in dioxan and phosphate buffer pH 8 was added. The mixture was left in a water bath at 37°C for 24 hrs. After the end of the hydrolysis the solvent was evaporated in vacuo and the residue was dried at 110°C in vacuum.

The well dried hydrolysis products were reduced by use of bis(4-methylpiperazinyl)aluminium hydride under reflux for 12 hrs. The resulting mixture of aldehydes was analysed with 2,4-dinitrophenylhydrazine gravimetrically.

When alcoholic potassium hydroxide was added to the obtained hydrazones, a stable purple colour was produced with absorption maximum at 556 nm, when it was measured after one hour. This purple colour was stable for several days. The relation between the absorption of the purple colour at 556 nm and the concentration of the hydrazones (µg/25 ml) is linear. The amount of the hydrazones was related to the amount of thalidomide which was hydrolysed previously.

It can be concluded that, at pH 8 the hydrolysis of thalidomide is very rapid, while an increase in pH from 7.4 to 8 doubled the rate of hydrolysis of the phthalimide ring, the rate of cleavage of the glutarimide ring was increased more than tenfold. The secondary hydrolysis products were appearing rapidly. The occurance of the band at 556 nm may be due to the overlapping of the band due to the hydrazone resulting from α-(o-carboxybenzamido)glutarimide, which remained without hydrolysis to phthalic acid.

Fig. 23 shows a Beer's law study of the hydrazones, obtained after the hydrolysis of thalidomide at pH 8 and 37°C for 24 hrs, and reduction of the hydrolysis products under reflux for 12 hrs.
Table 16 gives the data with which Fig. 24 was plotted.

Table 16

Concentration µg/25 ml	Absorption at 274 nm	Absorption at 428 nm	Absorption at 556 nm
180	0.260	0.1750	0.1885
360	0.543	0.3510	0.3720
540	0.870	0.5632	0.5950
720	1.190	0.7630	0.8126
900	1.460	0.9550	0.9970

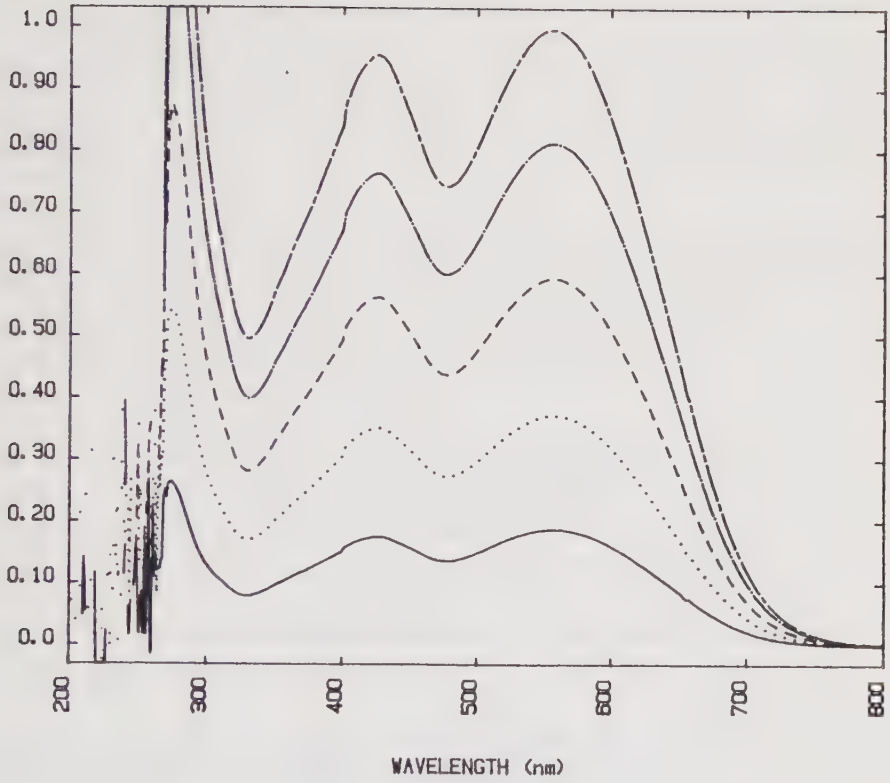

Fig. 23: Absorption spectra of five solutions of the hydrazones obtained from thalidomide (after its hydrolysis at pH 8, 37°C, 24 hrs and reduction under reflux) in methanolic potassium hydroxide solution.

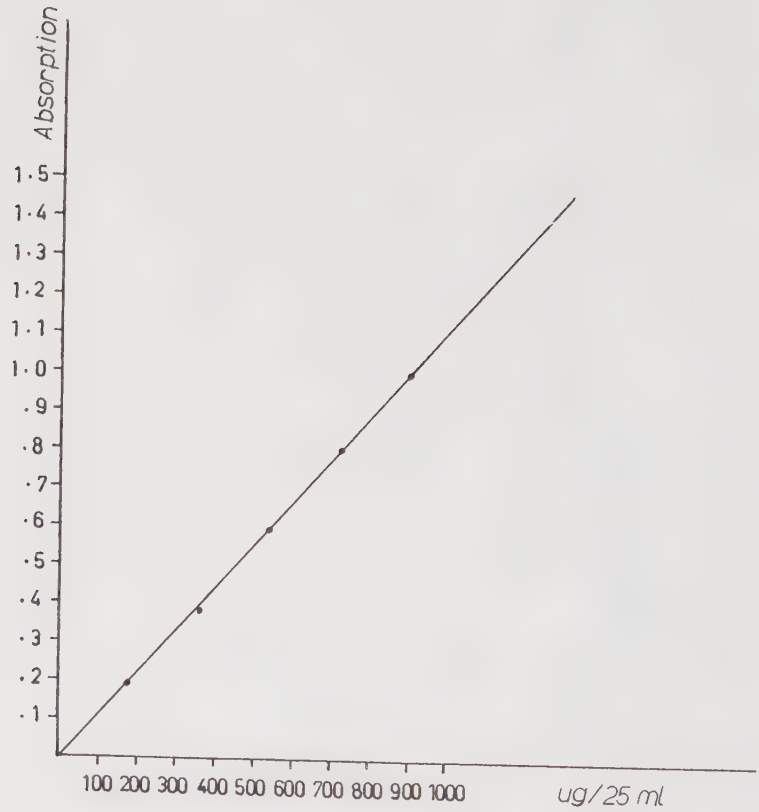

Fig. 24: Beer's law plot for the hydrazones obtained from thalidomide (hydrolysed at pH 8, 37°C, 24 hrs and reduced under reflux) in methanolic potassium hydroxide solution; data from Fig. 23 and Table 16 (λ_{max} = 556 nm).
y = 0.00114X - 0.0242
r = 0.9993
36 mg Hydrazones ≙ 24 mg Thalidomide

3.3.12. Quantitative Colour Reaction of Thalidomide, Hyrolysed at pH 8 and 37°C for 4 Hours

A few milligrams of thalidomide were dissolved in dioxan and phosphate buffer pH 8 was added. The mixture was left in a water bath at 37°C for 4 hrs. After the end of hydrolysis, the solvent was evaporated in vacuo and the residue was dried at 110°C in vauum.

The well dried mixture of hydrolysis products was reduced by use of bis(4-methylpiperazinyl)aluminium hydride under reflux for 12 hrs. The resulting mixture of aldehydes was analysed gravimetrically with 2,4-dinitrophenylhydrazine.

When alcoholic potassium hydroxide was added to the obtained hydrazones, a stable purple colour was produced with absorption maximum at 554 nm, when it was measured after one hour. Once again this colour was stable for several days. The relation between the absorption of the purple colour at 554 nm and the concentration of the hydrazones (µg/25 ml) is linear. The amount of the hydrazones was related to the amount of thalidomide previously hydrolysed.

Once again in this case of hydrolysis of thalidomide and reduction of its hydrolysis products, the colour obtained and its absorption maximum was similar as in case of the hydrolysis products of thalidomide for 24 hrs. It can be concluded, that all hydrolysis products of thalidomide, which occured after 24 hrs of hydrolysis at pH 8 were also occuring after 4 hrs hydrolysis at pH 8.

Figure 25 shows a Beer's law study of the hydrazones, obtained after the hydrolysis of thalidomide at pH 8 and 37°C for 4 hrs, and reduction of the hydrolysis products under reflux for 12 hrs.
Table 17 gives the data with which Fig. 26 was plotted.

Table 17

Concentration µg/25 ml	Absorption at 275 nm	Absorption at 426 nm	Absorption at 554 nm
196	0.362	0.2525	0.2539
392	0.732	0.4960	0.5081
588	1.080	0.7470	0.7580
784	1.430	0.9930	0.9970
1176	2.090	1.4900	1.4900

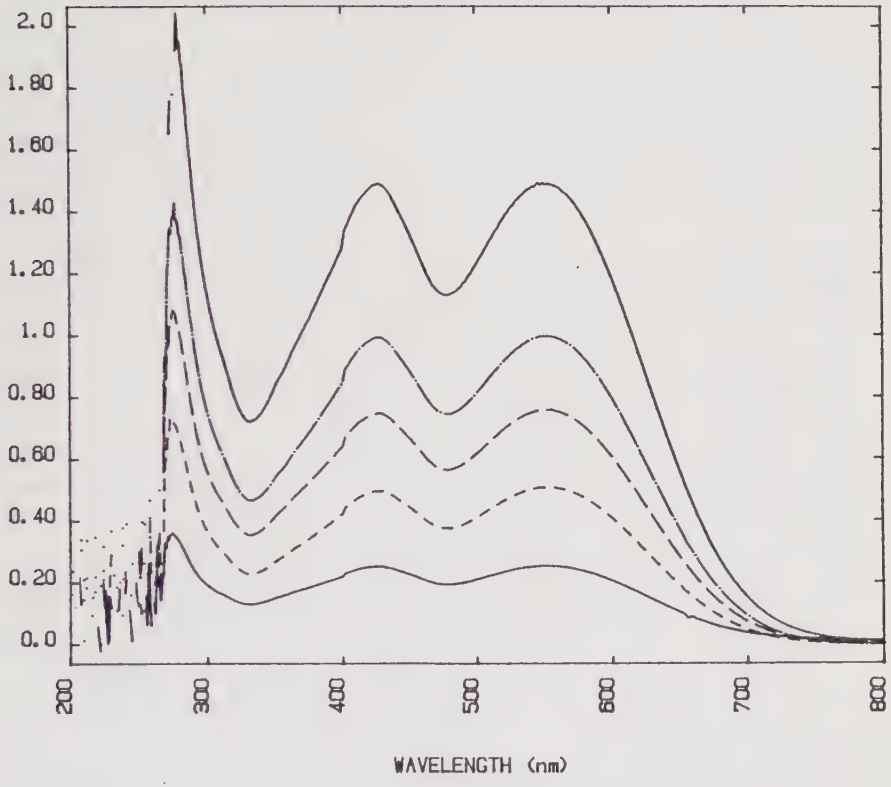

Fig. 25: Absorption spectra of five solutions of the hydrazones obtained from thalidomide (after its hydrolysis at pH 8, 37°C, 4 hrs and reduction under reflux) in methanolic potassium hydroxide solution.

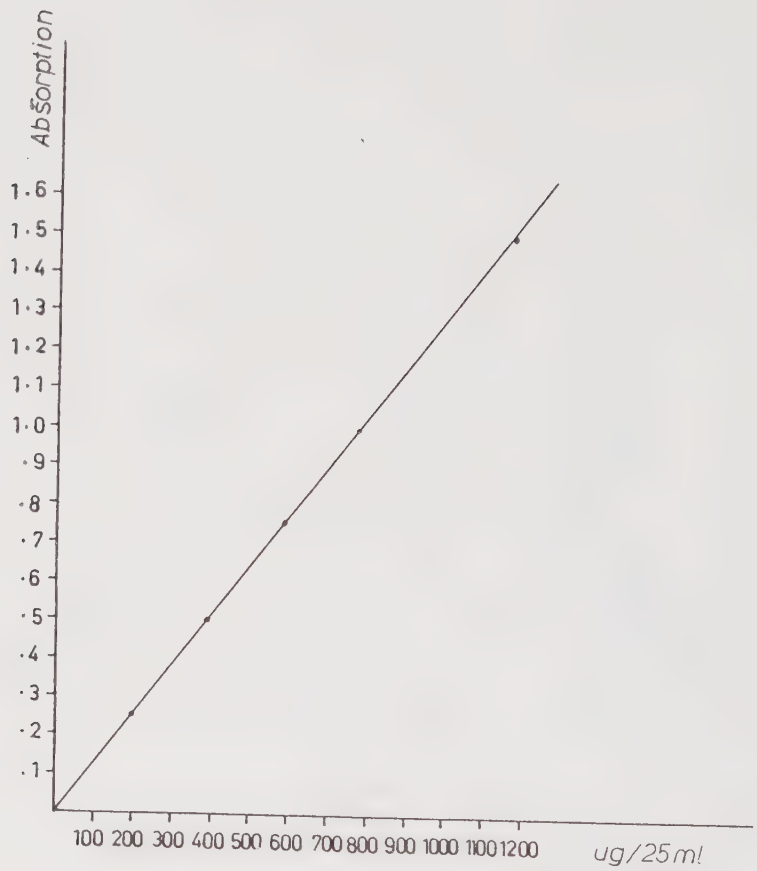

Fig. 26: Beer's law plot for the hydrazones obtained from thalidomide (hydrolysed at pH 8, 37°C, 4 hrs and reduced under reflux) in methanolic potassium hydroxide solution; data from Fig. 25 and Table 17 (λ_{max} = 554 nm).

y = 0.00158X + 0.0122757

r = 0.9999

39.2 mg Hydrazones ≙ 28 mg Thalidomide

3.3.13. Quantitative Colour Reaction of Thalidomide, Hydrolysed at pH 6 and 37°C for 24 Hours

A few milligrams of thalidomide were dissolved in dioxan and phosphate buffer pH 6 was added. The mixture was left in a water bath at 37°C for 24 hrs. After the end of hydrolysis, the solvent was evaporated in vacuo and the residue was dried in vacuum at 110°C.

The well dried mixture of hydrolysis products was reduced by use of bis(4-methylpiperazinyl)aluminium hydride under reflux for 12 hrs. The resulting mixture of aldehydes was analysed gravimetrically with 2,4-dinitrophenylhydrazine.

When alcoholic potassium hydroxide was added to the obtained hydrazones, a purple colour was produced. This colour remained for a very short time and then changed to a stable blue colour with absorption maximum at 618 nm, when it was measured after one hour. The blue colour was stable for several days. The relation between the absorption of the blue colour at 618 nm and the concentration of the hydrazones (µg/25 ml) is linear. The amount of the hydrazones was related to the amount of thalidomide, which was previously hydrolysed.

Figure 27 shows a Beer's law study of the hydrazones obtained after the hydrolysis of thalidomide at pH 6 and 37°C for 24 hrs and reduction of the hydrolysis products under reflux for 12 hrs.

Table 18 gives the data with which Fig. 28 was plotted.

Table 18

Concentration µg/25 ml	Absorption at 229 nm	Absorption at 271 nm	Absorption at 426 nm	Absorption at 618 nm
125	0.2330	0.2830	0.1100	0.1234
250	0.4304	0.3924	0.2140	0.2610
375	0.6924	0.6025	0.3282	0.3947
500	0.9610	0.8220	0.4460	0.5360
625	1.2300	1.0270	0.5595	0.6678

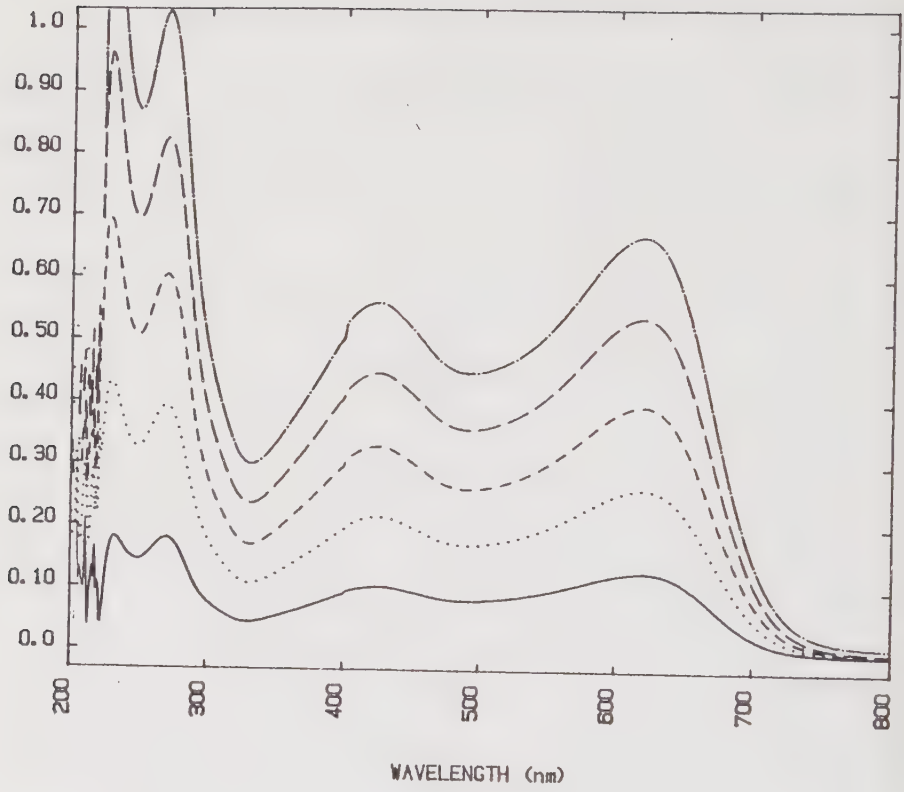

Fig. 27: Absorption spectra of five soutions of the hydrazones obtained from thalidomide (after its hydrolysis at pH 6, 37°C, 24 hrs and reduction under reflux) in methanolic potassium hydroxide solution.

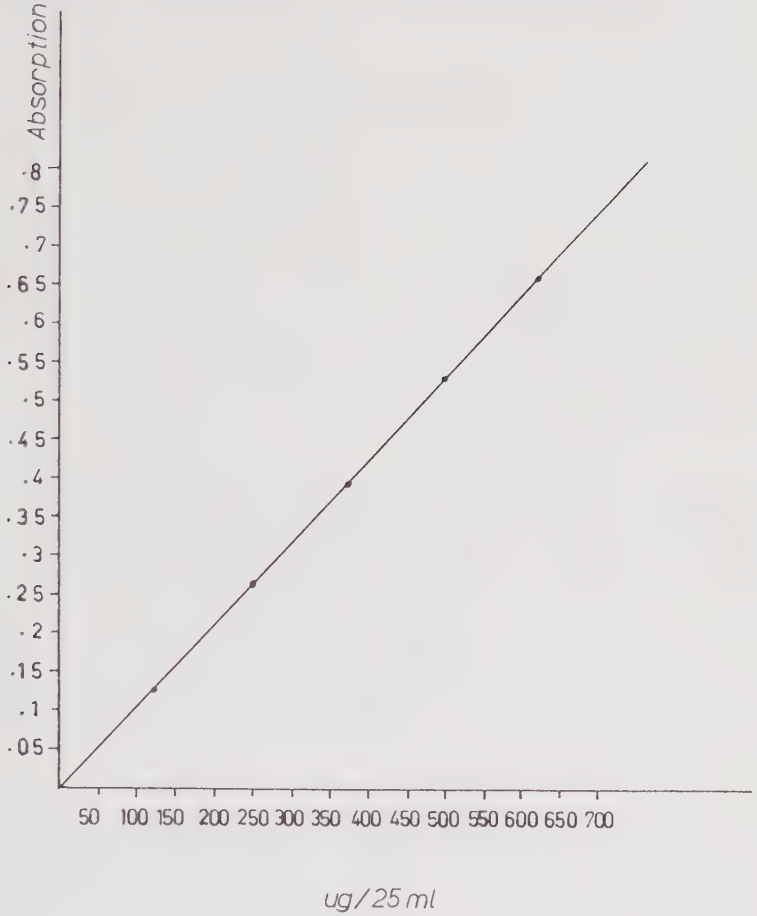

Fig. 28: Beer's law plot for the hydrazones obtained from thalidomide (hydrolysed at pH 6, 37°C, 24 hrs and reduced under reflux) in methanolic potassium hydroxide solution; data from Fig. 27 and Table 18 (λ_{max} = 618 nm).
y = 0.00109X - 0.01256
r = 0.9999
25 mg Hydrazones ≙ 38 mg Thalidomide

3.3.14. The Most Favourable Conditions for the Analysis of Thalidomide According to the Obtained Results

It was found in previous studies on thalidomide after its hydrolysis at different pH values, that the desired blue colour as a quantitative determination for thalidomide was dependent on the pH value at which thalidomide was hydrolysed, the time of hydrolysis and the conditions of the reduction of the mixture of hydrolysis products with bis(4-methylpiperazinyl)aluminium hydride. As the pH of the hydrolysis of thalidomide was increased from 6, 7.4 to 8, the blue colour was not obtained after the hydrolysis of thalidomide at pH 8, while it was obtained after the hydrolysis at pH 6 and 7.4.

Concerning the time of hydrolysis of thalidomide at pH 8, it was found, that the same purple colour with nearly the same position of the absorption maximum at 556 nm and 554 nm was obtained, when thalidomide was hydrolysed at pH 8 for 24 hrs and 4 hrs respectively. This indicated, that the hydrolysis products of thalidomide which occured after 24 hrs of hydrolysis at pH 8 were also found after 4 hrs hydrolysis at the same pH value and again this indicated, that the hydrolysis of thalidomide at pH 8 was very rapid.

After the hydrolysis of thalidomide at pH 7.4 for 24 hrs or 4 hrs the blue colour was obtained with the same position of the absorption maximum at 622 nm in case of the hydrolysis of thalidomide at pH 7.4 for 24 hrs then reduction of the obtained mixture of hydrolysis products at room temperature; and in case of the hydrolysis of thalidomide at pH 7.4 for 4 hrs and reduction of the obtained mixture of hydrolysis products under reflux for 12 hrs. The blue colour shows sharp hypsochromic shift (from 622 nm to 582 nm) when the obtained mixture of hydrolysis products (hydrolysis at pH 7.4 for 24 hrs) was reduced under reflux for 12 hrs, this may be due to the destruction of the hydrolysis products at this conditions of reduction.

Because the hydrolysis of thalidomide at pH 6 was very slow, it was only hydrolysed at this pH value for 24 hrs and after the reduction of the obtained mixture of hydrolysis products under reflux for 12 hrs, the blue colour was obtained with absorption maximum at 618 nm.

Thus, the favourable pH values for hydrolysis of thalidomide in order to obtain blue colour as a quantitative determination for thalidomide were pH 6 for 24 hrs and the physiological pH 7.4 for 4-24 hrs. But in regard to the value of absorption in relation to the quantity of the hydrazones which gave this value of absorption in comparison to the results obtained in 3.3.8., it can be said that the hydrolysis of thalidomide at pH 7.4 for 4 hrs gave the most sensitive conditions for the quantitative determination of thalidomide retardless to the very small value pathochromic shift from 618 nm in 3.3.8. to 622 nm when thalidomide was hydrolysed at pH 7.4 for 4 hrs.

3.4. Applications on Small Weight Quantities of Thalidomide Hydrolysed at Different pH Values

In this applications the samples should contain very small concentrations of aldehydes. In such dilute solutions the phenylhydrazones will not precipitate at room temperature. The solutions must be neutral or weakly acidic to prevent precipitation of potassium salts when the base is added.

To the sample solution in carbonyl-free methanol 2,4-dinitrophenylhydrazine reagent and a drop of concentrated hydrochloric acid were added. The mixture was heated in a water bath and after cooling potassium hydroxide solution was added. The almost black solution that resulted rapidly, cleared to the characteristic colour which changed to blue or purple. This colour was well developed after standing for one hour. A blank determination was made using the carbonyl-free methanol in place of the sample solution.

The optical density of the solution was determined using a Perkin-Elmer Spectronic 505. The instrument was adjusted for 100% transmittance for the solution from the blank determination. No further corrections for the blank were necessary.

The constructed graphs allowed direct reading of the hydrazone concentration from the observed optical density. This hydrazone concentration was related to the amount of thalidomide which was originally present as taken one and hydrolysed at different pH values and different periods. The obtained results are shown in Table 19.

Table 19

Taken amount of thalidomide (mg)	pH of Hydr.	Time of Hydr.	Condition of reduc.	Absorp. (nm)	Calculated amount of hydrazone (µg/25 ml)	Calculated amount of hydrazone (mg)	Found amount of thalidomide (mg)	% recovery
26.87	7.4	24 hrs	stirr. at RT 2 days	2.072 (622)	2192.500	21.92500	26.8580	99.96
12.93	7.4	24 hrs	ref. 12 hrs	2.103 (582)	1933.874	19.33874	12.9330	100.02
13.39	7.4	4 hrs	ref. 12 hrs	1.977 (622)	1196.347	11.96347	13.3620	99.79
10.85	8	24 hrs	ref. 12 hrs	1.826 (556)	1622.980	16.2298	10.8199	99.72
8.96	8	4 hrs	ref. 12 hrs	1.989 (554)	1251.090	12.5109	8.9364	99.74
28.58	6	24 hrs	ref. 12	2.032 (618)	1875.740	18.7574	28.5110	99.76

4. SUMMARY

The presented work deals with a quantitative colour reaction for thalidomide via its hydrolysis products, by the use of a spectrophotometric absorption technique.

4.1. In order to develop a quantitatively measureable colour, it was necessary to reduce the carboxylic acids which were obtained after the hydrolysis of thalidomide to aldehydes by the use of bis(4-methylpiperazinyl)aluminium hydride and conversion of the obtained aldehydes to the corresponding 2,4-dinitrophenylhydrazone derivatives. When alcoholic potassium hydroxide was added to the obtained hydrazones, a quantitative measurable blue colour was obtained according to the pH and time of hydrolysis.

4.2. Before working on thalidomide itself, the method was applied to N-methylphthalamic acid and some of the main hydrolysis products of thalidomide which are α-(o-carboxybenzamido)glutarimide, 2-phthalimidoglutaramic acid, 4-phthalimidoglutaramic acid, 2-phthalimidoglutaric acid and 2-(o-carboxybenzamido)glutaric acid. In all these examples, a calibration curve relating the concentration of the obtained 2,4-dinitrophenylhydrazone derivatives and the absorption of the developed colour after addition of alcoholic potassium hydroxide was plotted.

4.3. The work on a mixture of the hydrolysis products of thalidomide was done first by mixing these hydrolysis products according to the percentage obtained by Beckmann in urine of man and also plotting a calibration curve.

4.4. A very small quantity of thalidomide was hydrolysed at pH 6 for 24 hrs; pH 7.4 for 4 and 24 hrs; and pH 8 for 4 and 24 hrs by use of a phosphate buffer. In each case a calibration curve relating the concentration of the obtained hydrazones and the absorption maximum of the colour developed from these hydrazones was plotted.

4.5. As application of this method as a quantitative colour reaction for thalidomide, small weight quantities of thalidomide were hydrolysed at different pH values for different times. According to the value of absorption of colour (which was developed in each case by a method for trace quantities of aldehydes without separation of the obtained hydrazones), the concentrations of the hydrazones were read directly from the constructed calibration curves or by the use of the regression equations for these curves. The read concentrations of the hydrazones were related to the amount of thalidomide which was originally present as weighed sample.

5. EXPERIMENTAL PART

5.1. General Declarations

- All quantitative measurements of colours were carried out using a 8450 A uv/vis Spectrophotometer, Hewlett Packard.

- All applications on the quantitative measurements of colours were carried out using a Perkin-Elmer Spectrophotometer 505.

- All melting points are uncorrected and were carried out using a melting point microscope "Reichert Austria".

- Microanalysis were made in the microanalytical center of the Institute of Organic Chemistry of the University of Bonn.

- IR (KBr disc) were carried out using a Beckman Spectrometer.

- NMR 60 measurements were carried out using a Varian T 60 Spectrometer.

- NMR 90 measurements were carried out in the Institute of Organic Chemistry of the University of Bonn.

- Mass spectra measurements were carried out in the Institute of Organic Chemistry of the University of Bonn.

5.2. Preparation of Reagents

5.2.1. Preparation of 0.1 Mole of a 0.5 M Solution of Thexylborane in Tetrahydrofuran

A 250-ml three-necked flask, equipped with a magnetic stirring bar, a thermometer and a side arm, capped by a rubber septum, was flushed with a stream of dry argon. After cooling to 0°C under slight argon pressure, the reaction flask was charged with 100 ml of 1 M borane·THF solution [67] and 100 ml of 1 M 2,3-dimethyl-2-butene in THF were transferred to the addition funnel. The olefin-THF solution was added dropwise to the BH_3·THF reagent while a temperature below 0°C was maintained by use of a cooling thermostate apparatus. The thexylborane solution was stirred at 0°C for another two hours, after which the reagent was ready for use [68].

5.2.2. Preparation of 0.71 M Aluminium Hydride Solution

By means of a hypodermic syringe, 100 ml 1.7 M lithium aluminium hydride in THF and 100 ml of THF were introduced into a 300-ml flask, fitted with an inlet port, rubber syringe cap and magnetic stirring bar and connected (when it is possible) to a gas meter via the reflux condenser. To the solution 8.35 g of 100% sulfuric acid (85.2 mmoles) were added slowly by means of a syringe, while stirring the solution vigorously.

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67) For a complete description of the techniques necessary in transferring the solutions, consult the bulletin "Handling Air Sensitive Solutions" supplied with borane-THF complex; Technical Information for the Product Nr. 18,525-6, EGA Firm (1983).

68) For general review on organoborane chemistry, see H.C.Brown: "Boranes in Organic Chemistry", Cornell University Press, Ithaca, N.Y., 1972 (Aldrich Catalog no. Z10, 080-3, $ 37.50).

The solution was permitted to stir for one hour and then allowed to stand at room temperature to permit the lithium sulfate precipitate to settle. The clear, supernatant solution was removed by a syringe.

5.2.3. Preparation of Bis(4-methylpiperazinyl)aluminium Hydride

N-methylpiperazine (14.1 g, 141 mmol) was added to a 0.71 M solution of aluminum hydride in anhydrous THF (97 ml, 69 mmol) over a period of 15 minutes at 0°C under argon. After stirring for two hrs at room temperature, a clear solution of bis(4-methylpiperazinyl)aluminium hydride resulted.

5.2.4. Carbonyl-Free Methanol

To 500 ml of CP methanol about 5 g of 2,4-dinitrophenylhydrazine and a few drops of concentrated hydrochloric acid were added. After refluxing for 2 hrs, the methanol was distilled through a short Vigreux column. If kept tightly stoppered, the methanol remains suitable for the use several months.

5.2.5. 2,4-Dinitrophenylhydrazine Solution

A saturated solution in carbonyl-free methanol was prepared, using 2,4-dinitrophenylhydrazine that had been recrystllized twice from this solvent. This solution should not be used more than a week or two after preparation.

5.2.6. Potassium Hydroxide Solution

Ten grams of potassium hydroxide were dissolved in 20 ml of distilled water and the solution was made up to 100 ml with carbonyl-free methanol. This solution was kept indefinitely.

5.2.7. Buffer Phosphate Solution

pH 6.0: 50 ml of 0.1 M potassium dihydrogen phosphate + 5.6 ml of 0.1 M sodium hydroxide.

pH 7.4: 50 ml of 0.1 M potassium dihydrogen phosphate + 39.1 ml of 0.1 M sodium hydroxide.

pH 8.0: 50 ml of 0.1 M potassium dihydrogen phosphate + 46.1 ml of 0.1 M sodium hydroxide.

5.2.8. Absolute Tetrahydrofuran

To two liters of THF were added 20 grams of Cu_2Cl_2. After refluxing for 2 hrs, the THF was distilled into a flask provided with $CaCl_2$ drying tube and charged with 20 g KOH. After refluxing once again for 2 hrs, the THF was distilled into a dropping funnel with pressure equalizing tube. The distillate was filtered over Al_2O_3 Act. 1 column and sodium metal was pressed into the filtrate.

5.3. N-Methylphthalamic Acid XIX [60]

3 g phthalic anhydride were treated with aqueous methylamine (30% 10 c.c.). The solution occured with considerable evolution of heat. The solution was cooled and carefully acidified with cold hydrochloric acid. After standing, the crystalline mass was collected and shaken with cold N-sodium hydroxide (10 ml). The insoluble portion was removed and shown to be N-methylphthalimide m.p. 181°C [60]. The alkaline solution was cooled with ice water and acidified with concentrated hydrochloric acid. On standing N-methylphthalamic acid separated as prisms, m.p. 137°C, totally soluble with effervescence in sodium bicarbonate solution (yield 1.252 g, 69%). For analysis the acid was repeatedly washed with cold distilled water and dried at room temperature under reduced pressure over potassium hydroxide ($C_9H_9O_3N$, M.wt. 179: Calc. C 60.33; H 5.03; N 7.82. Found: C 59.79; H 5.003; N 7.855); IR and ^{1}H.n.m.r. p. 101).

5.3.1. Reduction of N-Methylphthalamic Acid by Use of Thexylborane

In a 100-ml double wall flask, equipped with a reflux condenser with P_2O_5 drying tube, magnetic stirring bar, dry argon gas inlet and thermometer were placed 25 ml of thexylborane solution in THF by use of a transferring syringe. The solution was then allowed to cool to -20°C. 0.895 g N-methylphthalamic acid in 5 ml absolute THF (cooled to -20°C) were added slowly under vigorous stirring. After 10 minutes the mixture was heated and kept under reflux. After different time intervals the mixture was cooled to 0°C and 5 ml aliquots were withdrawn and analysed for aldehyde as in 5.3.2.. After 12 hrs 28.6 mg of 2,4-DNPH derivative were obtained (10%); after 24 hrs 62.92 mg (22%) were obtained; and this remained constant after 36 hrs.

5.3.2. 2,4-Dinitrophenylhydrazine Test Solution

In a 100-ml Erlenmeyer flask 0.4 g of 2,4-dinitrophenylhydrazine were dissolved in a mixture of 2 ml of concentrated sulfuric acid and 3 ml of water. To this solution 5 ml of carbonyl-free methanol were added, followed by the addition of 5 ml of the reaction mixture under investigation. After a short time, the expected 2,4-dinitrophenylhydrazone crystallized. After 15 minutes, 10 ml of water were added and the mixture was allowed to stand for 1 hr at room temperature. The crystals were separated from the liquid by a glass filter and washed several times with 2N hydrochloric acid, followed by a mixture of water/carbonyl-free methanol (3:1). The substance was dried over concentrated sulfurc acid in vacuum to constant weight.

5.3.3. Reduction of N-Methylphthalamic Acid by Use of Bis(4-methylpiperazinyl)aluminium Hydride

In a 50-ml double wall flask, equipped with a reflux condenser with P_2O_5 drying tube, stirring bar, dry argon inlet and thermometer were placed 20 ml of bis(4-methylpiperazinyl)-aluminium hydride by use of a transferring syringe. The solution was allowed to cool to 0°C. 400 mg of N-methylphthalamic acid in 5 ml absolute THF cooled to 0°C were added slowly with vigorous stirring. The solution was refluxed for 6 hrs, then water (0.5 ml) was added to the solution at 0°C, followed by the addition of 5 ml carbonyl-free methanol. Crystals were filtered off and extracted repeatedly with carbonyl-free methanol. The filtrate and the extracts were condensed in vacuo to nearly 5 ml. This residue was analysed for aldehyde content as in 5.3.2.. After 6 hrs reflux 321.78 mg of 2,4-DNPH derivative were obtained (42%). The above reduction procedure was repeated for 10, 12 and 14 hrs. After 10 hrs 436.7 mg (57%) were obtained; after 12 hrs 498 mg (65%) were obtained and this remained constant after 14 hrs, m.p. 118°C.

Microanalysis: $C_{15}H_{13}N_5O_5$, M.wt. 343: Calc. C 52.47; H 3.79; N 20.4. Found: C 52.82; H 3.86; N 20.48.
In mass spectrum this hydrazone shows a molecular ion peak at 343, p. 102.

5.3.4. Quantitative Measurement of the Colour Developed from the Foregoing Hydrazone

4.15 mg of the obtained hydrazone, after the reduction of N-methylphthalamic acid, were dissolved in 10 ml methanolic potassium hydroxide reagent. From this solution, samples of 0.5, 1, 1.5, 2 and 2.5 ml were withdrawn by use of a micropipette and each sample was diluted up to 25 ml with carbonyl-free methanol. After standing for one hr, the measurement of absorption of colour was begun starting with the lowest concentration up to the highest. The results obtained were shown in Table 5, Figure 3 and Figure 4.

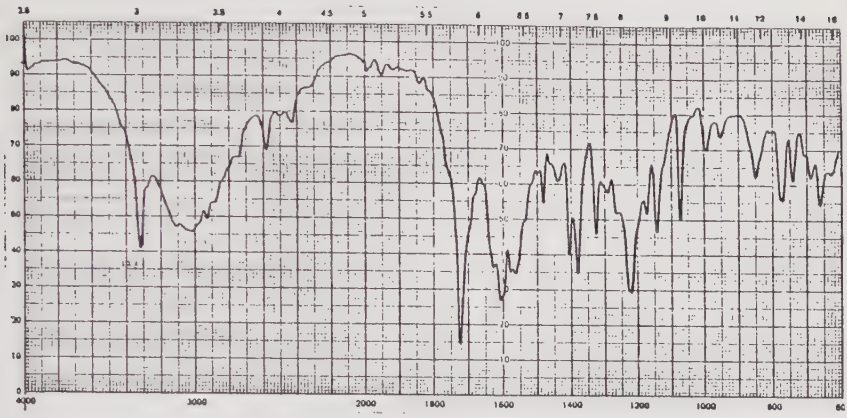

Fig. 29: IR spectrum of N-methylphthalamic acid
3320 (NH); 2580 (COOH); 1720 (C=O).

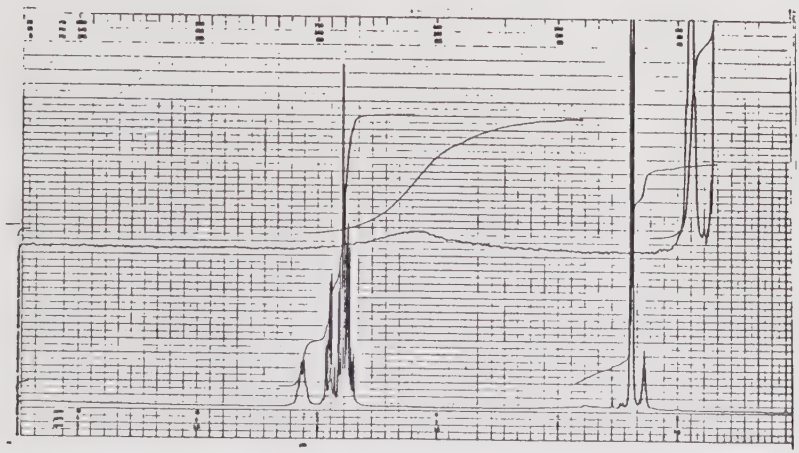

Fig. 30: ^{1}H.n.m.r. spectrum of N-methylphthalamic acid in DMSO-d_6: 2.76 (d, 3H, CH_3); 8.27 (q, 1H, NH, "a"); 7.62 (m, 4H, aromatic); 13.12 (s, 1H, COOH, "a").

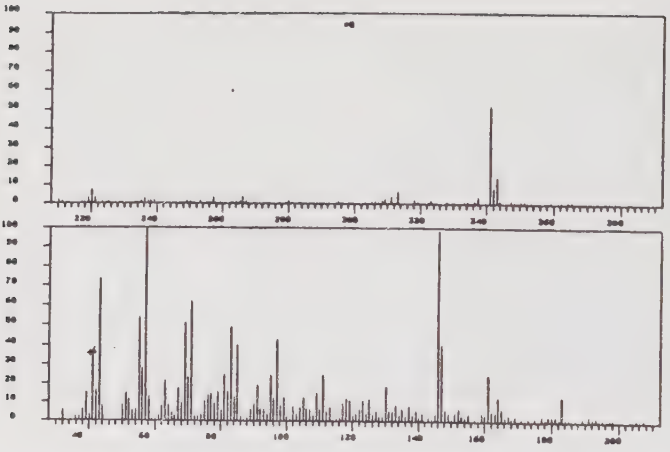

Fig. 31: Mass spectrum of the hydrazone obtained from N-methylphthalaldehyde (with molecular ion peak at 343).

5.4. D,L-α-Aminoglutarimide Hydrochloride (X) [63]

31 g (0.12 mole) thalidomide (Chemistry Grünenthal GmbH) was suspended in 150 ml of pyridine. 6.5 g (0.13 mole) hydrazine hydrate were added and the mixture was stirred occasionally for 2 hrs. After that a clear yellowish solution was obtained. The pyridine was evaporated in vacuo at 50°C and 150 ml of hydrochloric acid 10% were added to the residue. The mixture was left under occasional stirring for 10 hrs at room temperature. The phthalic acid hydrazide precipitated was filtered off and the filtrate was dried in vacuo. The residue was dissolved in 150 ml of water at 40°C. The mixture was filtered and to the clear filtrate 800 ml ethanol were added. The filtrate was left at 0°C for 24 hrs. On standing D,L-α-aminoglutarimide hydrochloride separated as crystals, which were dried at 60°C, m.p. 200°C (decomp.), yield 42%.
Microanalysis: $C_5H_9N_2O_2Cl$, M.wt. 164.5:
Calc. C 36.49; H 5.51; N 17.02; Cl 21.54.
Found: C 36.57; H 5.57; N 17.18; Cl 21.73.
The mass spectrum shows a molecular ion peak at 128 and HCl at 36.5, p. 106.

5.4.1. α-(o-Carboxybenzamido)glutarimide (IV) [28]

4.9 g (30 mmole) D,L-α-aminoglutarimide hydrochloride were dissolved in methanol/water (1:4). After addition of 25 ml ionexchanger Amberlite IRA 68 (OH^--form), which was suspended in water, the mixture was shaken for 5 minutes and then filtered. The ionexchanger was washed with water/methanol and the combined filtrates were dried in vacuo at 40°C. The crystalline residue of the free D,L-α-aminoglutarimide was rapidly reacted because of its instability. The dry crystalline residue was suspended in dry pyridine and 4.4 g (30 mmole) of phthalic anhydride were added and the mixture was stirred at room temperature over night. The pale violet coloured solution was evaporated in vacuo at 35°C and the oily residue was taken

in 25 ml of water, then acidified to pH 2 with 2N hydrochloric acid. The violet coloured precipitate was filtered off and washed with 2N hydrochloric acid, then it was dissolved in 1N sodium bicarbonate solution and the solution was decolourized with charcoal, filtered and then acidified with 2N hydrochloric acid. The crystals of α-(o-carboxybenzamido)glutarimide, which were obtained had m.p. 271°C after loss of water at 200°C converted to thalidomide, yield: 57%.

Microanalysis: Calc.: C 56.52; H 4.38; N 10.14.
Found: C 56.14; H 4.43; N 10.17.

5.4.2. Reduction of α-(o-Carboxybenzamido)glutarimide by Use of Bis(4-methylpiperazinyl)aluminium Hydride

In a 50-ml double wall flask, equipped with a reflux condenser (with P_2O_5 drying tube), stirring bar, dry argon inlet and thermometer were placed 15 ml of bis(4-methylpiperazinyl)-aluminium hydride by use of a transferring syringe. The solution was allowed to cool to 0°C and 0.4 g (1.5 mmole) of α-(o-carboxybenzamido)glutarimide in 5 ml absolute THF were added slowly with vigorous stirring. The solution was refluxed for 12 hrs. 0.7 ml of water were added to the solution at 0°C, followed by 5 ml of carbonyl-free methanol. Crystals were filtered off and extracted repeatedly with carbonyl-free methanol. The filtrate and the extracts were condensed in vacuo to about 5 ml. This residue was analysed for aldehyde content as in 5.3.2.. After 12 hrs reflux 333.73 mg of the 2,4-DNPH derivative were obtained (70%). In mass spectrum this hydrazone shows a molecular peak ion at 329, m.p. 115°C.

Microanalysis: $C_{14}H_{11}N_5O_5$, M.wt. 329.
Calc.: C 51.06; H 3.34; N 21.28.
Found: C 51.32; H 3.39; N 21.42.

5.4.3. Quantitative Measurement of the Colour Developed from the Foregoing Hydrazone

7.7 mg of the obtained hydrazone, after reduction of α-(o-carboxybenzamido)glutarimide were dissolved in 25 ml of methanolic potassium hydroxide reagent. From this solution samples of 0.5, 1, 1.5, 2 and 2.5 ml were withdrawn by the use of a micropipette and each sample was diluted up to 25 ml with carbonyl-free methanol. After standing for one hr, the measurements of the absorption of colours were begun starting as usual with the measurement of the lowest concentration up to the highest one. The results obtained are shown in Table 6, Figure 5 and Figure 6.

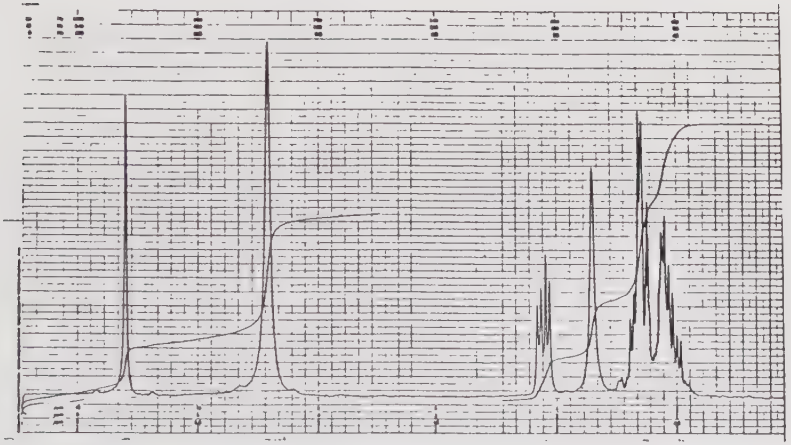

Fig. 32: ^{1}H.n.m.r. spectrum of α-aminoglutarimide hydrochloride in DMSO-d_6: 2.11 (m, 2H, CH_2); 2.66 (m, 2H, CH_2); 4.22 (q, 1H, CH); 8.84 (s, 3H, NH_2 + HCl, "a"); 11.22 (s, 1H, NH, "a").

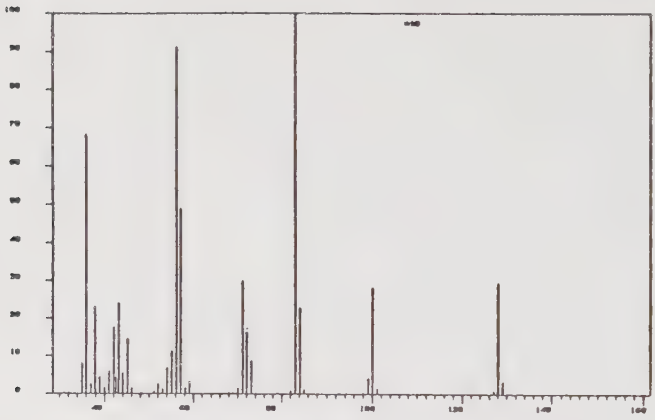

Fig. 33: Mass spectrum of α-aminoglutarimide hydrochloride (with molecular ion peak at 128 + HCl at 36.5).

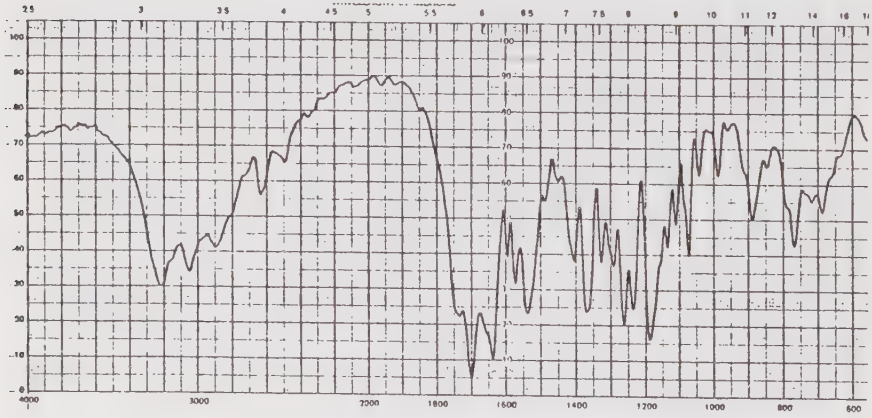

Fig. 34: IR spectrum of α-(o-carboxybenzamido)glutarimide 3220 (NH); 2640 (COOH); 1700-1640 (C=O).

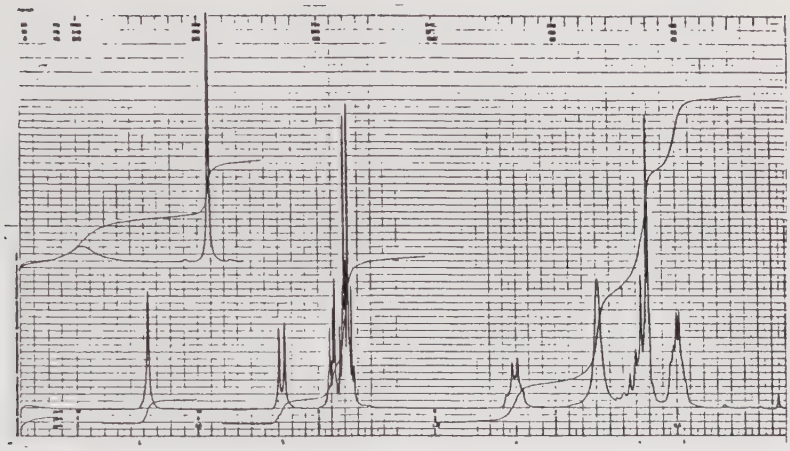

Fig. 35: ^{1}H.n.m.r. spectrum of α-(o-carboxybenzamido)glutarimide in DMSO-d_6: 2.0 (m, 2H, CH_2); 2.6 (m, 2H, CH_2); 4.66 (m, 1H, CH); 7.62 (m, 4H, aromatic); 8.57 (d, 1H, NH, "a"); 10.82 (s, 1H, COOH, "a"); 12.70 (s, 1H, NH, "a").

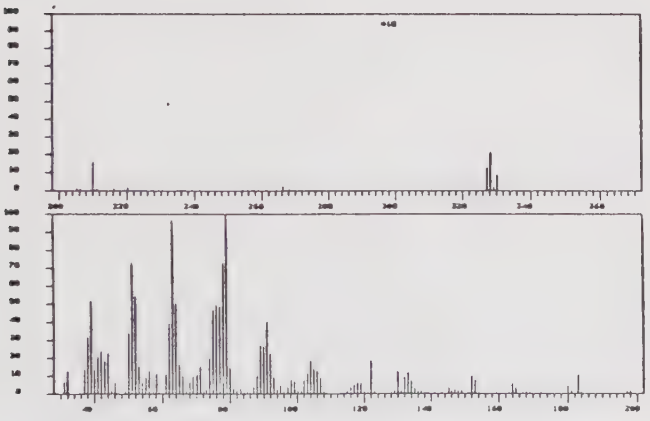

Fig. 36: Mass spectrum of the hydrazone obtained from α-(o-carboxybenzamido)glutarimide (with molecular ion peak at 329).

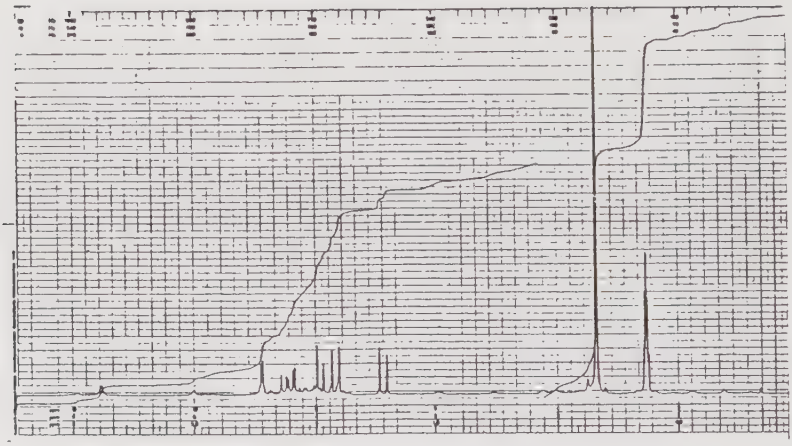

Fig. 37: ^{1}H.n.m.r. spectrum of the hydrazone obtained from α-(o-carboxybenzamido)glutarimide in DMSO-d_6: 6.88 (2s, <2H, CH_2, equilibrium); 8.05 (m, 7H, aromatic); 10.0 (s, <1H, NH, "a", equilibrium); 11.73 (s, 2H, NH_2, "a").

5.5. Phthalyl-D,L-glutamic Anhydride [64]

A suspension of 65.2 g L-glutamic acid and 65.2 g phthalic anhydride in 240 ml dry pyridine was refluxed for 2 hrs. After evaporation of the clear solution under reduced pressure, 180 ml acetic anhydride were added and the mixture was boiled for 5 minutes. Crystallization of the product began during concentration of the solvent at diminished pressure and was completed on cooling by cautious addition of anhydrous ether. After being washed with ether and dried, the phthalyl-D,L-glutamic anhydride had m.p. 197°C, yield 78.8 g; 72%.

5.5.1. 2-Phthalimidoglutaramic Acid (III) [64]

A solution of phthalyl-D,L-glutamic anhydride (15 g) in warm dioxan (120 ml) was cooled in water and treated with small quantities of dry ethereal ammonia in excess. When the precipitation was complete the bulky ammonium salt was collected, washed with ether and dissolved in a little water. On acidification (Congo-red) with 5N hydrochloric acid (11.4 g; 72%) slowly separated and after several hours in refrigerator were collected and recrystallized from water, giving small colourless prisms m.p. 196°C.

Microanalysis: $C_{13}H_{12}N_2O_5$, M.wt. 276:
Calc.: C 56.52; H 4.38; N 10.14.
Found: C 56.31; H 4.45; N 9.77.

5.5.2. Reduction of 2-Phthalimidoglutaramic Acid by Use of Bis(4-methylpiperazinyl)aluminium Hydride

In a 100-ml double wall flask, equipped with a reflux condenser (with P_2O_5 tube), stirring bar, dry argon inlet and thermometer 25 ml of bis(4-methylpiperazinyl)aluminium hydride were placed by use of a transferring syringe. The solution was allowed to cool to 0°C. 0.7 g (2.54 mmole) of 2-phthalimidoglutaramic acid in 10 ml absolute THF (cooled to 0°C) were added slowly with vigorous stirring. The solution was refluxed for 12 hrs. Then water (1.4 ml) was added to the solution at 0°C followed by 8 ml of carbonyl-free methanol. Crystals were filtered off and extracted repeatedly with carbonyl-free methanol. The filtrate and the extracts were condensed in vacuo to about 5 ml. This residue was analysed for aldehyde content as in 5.3.2.. After 12 hrs reflux 781.13 mg of 2,4-DNPH derivative were obtained (70%), m.p. 78°C.

Microanalysis: $C_{19}H_{16}N_6O_7$, M.wt. 440.
Calc.: C 51.82; H 3.64; N 19.1.
Found: C 51.97; H 3.77; N 19.21.
In the mass spectrum the molecular ion peak is unstable and shows a very small M+1 peak at 441, p. 116.

5.5.3. Quantitative Measurement of the Colour Developed from the Foregoing Hydrazone

14 mg of the obtained hydrazone, after reduction of 2-phthalimidoglutaramic acid, were dissolved in 50 ml methanolic potassium hydroxide reagent. From this solution samples of 1, 2, 3, 4 and 5 ml were withdrawn and each sample was diluted up to 25 ml with carbonyl-free methanol. After standing one hour the measurement of the absorption of colour was begun starting with the lowest concentrated sample to the highest one. The results are shown in Table 7, Figure 7 and Figure 8.

5.6. γ-Benzyl Hydrogen Phthalyl-D,L-Glutamate [65]

Phthalyl-D,L-glutamic anhydride (8 g) was heated with benzyl alcohol (12 g) at 100°C until a clear solution was obtained. Then ether was added and the ethereal solution was extracted with sodium hydrogen carbonate. The combined extracts were washed with ether and acidified with hydrochloric acid, the precipitated oil being taken up in ether. Drying and evaporation of the ethereal solution left a gum (9.9 g) which solidified in contact with benzene and crystallization from benzene/light petroleum gave γ-benzyl hydrogen phthalyl-D,L-glutamate as colourless soft needles, m.p. 83°C.

5.6.1. Phthalyl-D,L-Isoglutamin Benzyl Ester [65]

A solution of the γ-benzyl hydrogen phthalyl-D,L-glutamate (6.7 g) in dry ether (50 ml) was shaken with phosphorous pentachloride (3.92 g) with occasional cooling in ice-water, until the reaction was complete. The pale yellow syrup left on removal of the ether was washed well with light petroleum and then redissolved in ether. Ethereal ammonia was added until

precipitation ceased and after collection, the solid was titurated with a dilute aqueous solution of sodium hydrogen carbonate. The residue of phthalyl-D,L-isoglutamine benzyl ester (5.8 g) crystallized from water, m.p. 124°C.

5.6.2. 4-Phthalimidoglutaramic Acid (II) [65]

The foregoing benzyl ester (4 g) in aqueous ethanol (100 ml) containing acetic acid (0.5 ml) was hydrogenated over palladium-charcoal at two atmospheres pressure and 60°C. The residue obtained after evaporating the filtered solution to dryness was shaken with an aqueous solution of sodium hydrogen carbonate and the soluble portion precipitated with hydrochloric acid. The deposited 4-phthalimidoglutaramic acid (2.2 g) crystallized from aqueous ethanol in fine needles, m.p. 176°C with shrinking at 100-120°C. On drying in vacuum at 110°C loss in weight occured due to the loss of water of crystallization.

Microanalysis of the dried material:

$C_{13}H_{12}N_5O_5$ M.wt. 276. Calc.: C 56.52; H 4.38; N 10.14.
Found: C 56.268; H 4.62, N 9.544.

5.6.3. Reduction of the Dried Material of 4-Phthalimidoglutaramic Acid by Use of Bis(4-methylpiperazinyl)aluminium Hydride

In a 100-ml double wall flask, equipped with a reflux condenser (with P_2O_5 drying tube), stirring bar, dry argon inlet and thermometer 22 ml of bis(4-methylpiperazinyl)aluminium hydride were placed by use of a transferring syringe. The solution was then allowed to cool to 0°C. 0.6 g (2.2 mmol) of 4-phthalimidoglutaramic acid (dried in vacuum at 110°C) in 10 ml of absolute THF (cooled to 0°C) were added slowly with vigorous stirring. The solution was refluxed for 12 hrs. Then water (1.2 ml) was added to the solution at 0°C followed by 7 ml of

carbonyl-free methanol. Crystals were filtered off and extracted repeatedly with carbonyl-free methanol. The filtrate and the extracts were condensed in vacuo to about 5 ml. This residue was analysed for the aldehyde content as in 5.3.2.. After 12 hrs reflux, 698.26 mg of 2,4-DNPH derivative were obtained (73%) m.p. 83°C.
Microanalysis: $C_{19}H_{16}N_6O_7$, M.wt. 440.
Calc.: C 51.82; H 3.64; N 19.1.
Found: C 52.02; H 3.87; N 19.42.

5.6.4. Quantitative Measurement of the Colour Developed from the Foregoing Hydrazone

3.2 mg of the obtained hydrazone after reduction of 4-phthalimidoglutaramic acid were dissolved in 10 ml of methanolic potassium hydroxide reagent. From this solution samples of 0.5, 1, 1.5, 2 and 2.5 ml were taken by the use of a micropipette and each sample was diluted up to 25 ml with carbonyl-free methanol. After standing for one hour the measurements of the absorption of colour were begun starting with the lowest concentration up to the highest. The results obtained are shown in Table 8, Figure 9 and Figure 10.

5.7. 2-Phthalimidoglutaric Acid (V) [64]

5 g of phthalylglutamic anhydride were dissolved in the minimum amount of boiling water; on cooling 2-phthalimidoglutaric acid crystallized in colourless needles, m.p. 190°C.
Microanalysis: $C_{13}H_{11}NO_6$, M.wt. 277.
Calc.: C 56.31; H 3.79; N 5.05.
Found: C 56.32; H 4.1; N 5.03.

5.7.1. Reduction of 2-Phthalimidoglutaric Acid by Use of Bis(4-methylpiperazinyl)aluminium Hydride

In a 50-ml double wall flask, equipped with a reflux condenser (with P_2O_5 drying tube), stirring bar, dry argon inlet and thermometer 27 ml of bis(4-methylpiperazinyl)aluminium hydride were placed by the use of a transferring syringe. The solution was allowed to cool to 0°C. 0.4 g (1.4 mmol) of 2-phthalimidoglutaric acid in 5 ml of absolute THF (cooled to 0°C) were added slowly with vigorous stirring. The solution was refluxed for 12 hrs. Then water (1.5 ml) was added to the solution at 0°C followed by 5 ml of carbonyl-free methanol. Crystals were filtered off and extracted repeatedly with carbonyl-free methanol. The filtrate and the extracts were condensed in vacuo to about 5 ml. This residue was analysed for aldehyde content as in 5.3.2.. After 12 hrs reflux 515.44 mg of 2,4-DNPH derivative were obtained, m.p. 83-85°C.

5.7.2. Quantitative Measurement of the Colour Developed from the Foregoing Hydrazone

12 mg of the obtained hydrazone after reduction of 2-phthalimidoglutaric acid were dissolved in 50 ml methanolic potassium hydroxide reagent. From this solution samples of 1, 2, 3 and 4 ml were taken and each sample was diluted up to 25 ml with carbonyl-free methanol. After standing for one hour the measurements of the absorption of colour were begun with the lowest concentrated sample to the highest one. The results are shown in Table 9, Figure 11 and Figure 12.

5.8. 2-(o-Carboxybenzamido)glutaric Acid (VIII) [28]

2.78 g (10 mmol) of 2-phthalimidoglutaric acid V were dissolved in 15 ml of 2N sodium hydroxide. After 10 minutes the solution was neutralized with formic acid and then filtered

over an ion exchanger column (Amberlite IR-120, H^+-form). The eluent was evporated in vacuo and the residue was recrystallized from water, 1.8 g (60.8%) were obtained, m.p. 163°C.
Microanalysis: $C_{13}H_{13}NO_7$, M.wt. 295.
Calc.: C 52.88; H 4.44; N 4.74.
Found: C 52.6; H 4.48; N 4.73.

5.8.1. Reduction of 2-(o-Carboxybenzamido)glutaric Acid by Use of Bis(4-methylpiperazinyl)aluminium Hydride

In a 100-ml double wall flask equipped as before, 40 ml of bis(4-methylpiperazinyl)aluminium hydride were placed by the use of a syringe. The solution was allowed to cool to 0°C. 0.4 g (1.4 mmol) of 2-(o-carboxybenzamido)glutaric acid in 5 ml of absolute THF (cooled to 0°C) were added slowly with vigorous stirring. The solution was refluxed for 12 hrs. Then water (2 ml) was added to the solution at 0°C follwoed by 5 ml of carbonyl-free methanol. Crystals were filtered off and extracted repeatedly with carbonyl-free methanol. The filtrate and extracts were condensed in vacuo to about 5 ml. This residue was analysed for aldehyde content as in 5.3.2.. After 12 hrs reflux 586.9 mg of 2,4-DNPH derivative were obtained, m.p. 71-73°C.

5.8.2. Quantitative Measurement of the Colour Developed from the Foregoing Hydrazone

9.7 mg of the obtained hydrazone after the reduction of 2-(o-carboxybenzamido)glutaric acid were dissolved in 50 ml of methanolic potassium hydroxide reagent. From this solution samples of 1, 2, 3, 4 and 5 ml were taken and each sample was diluted up to 25 ml with carbonyl-free methanol. After standing for one hour, the measurements of the absorption of colour started with the lowest concentrated sample continuing to the highest concentrated one. The results obtained are shown in Table 10, Figure 13 and Figure 14.

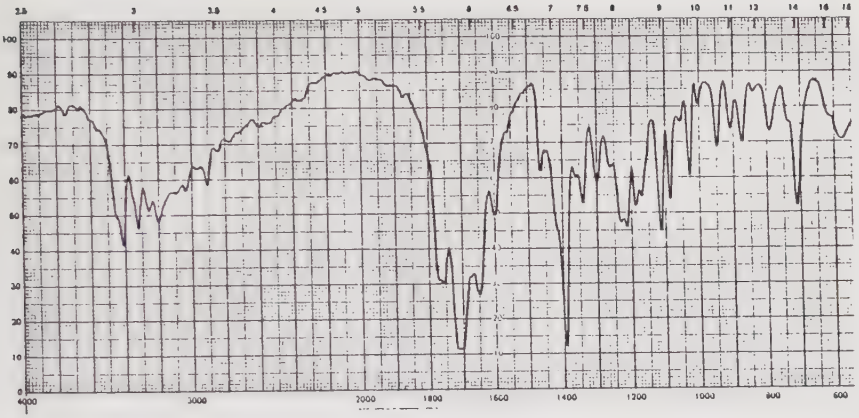

Fig. 38: IR spectrum of 2-phthalimidoglutaramic acid
3410 (NH); 2850 (COOH); 1760-1650 (C=O).

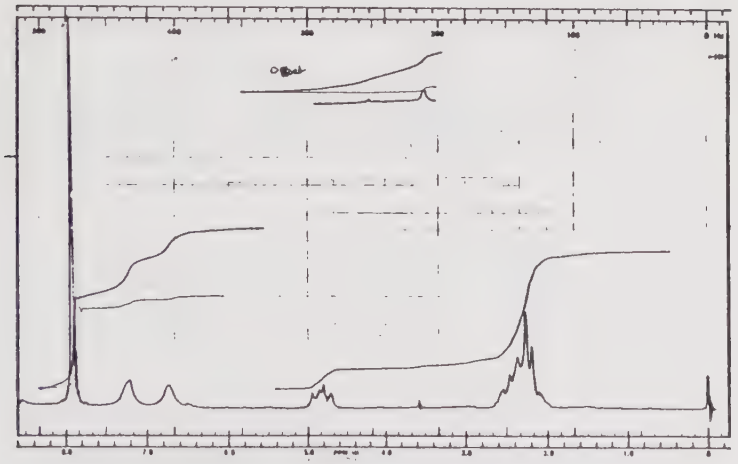

Fig. 39: ^{1}H.n.m.r. of 2-phthalimidoglutaramic acid in DMSO-d_6:
2.33 (m, 4H, 2 CH_2); 4.83 (m, 1H, CH); 7.06 (d, 2H,
NH_2, "a"); 7.93 (s, 4H, aromatic); 8.66 (s, 1H, COOH,
"a").

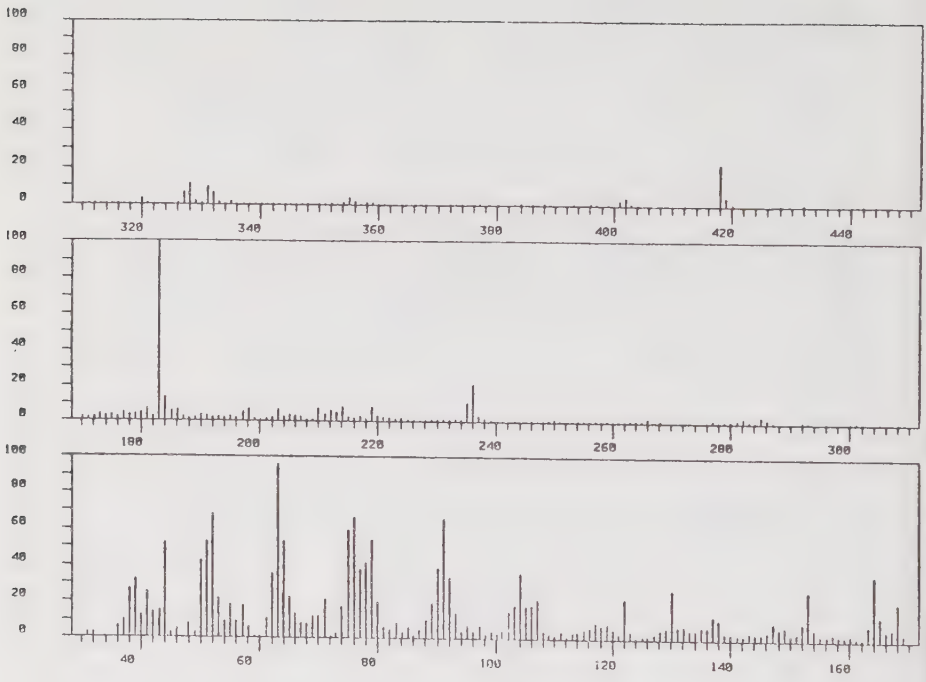

Fig. 40: Mass spectrum of the hydrazone of 2-phthalimidoglutaraldehyde with M+1 peak at 441.

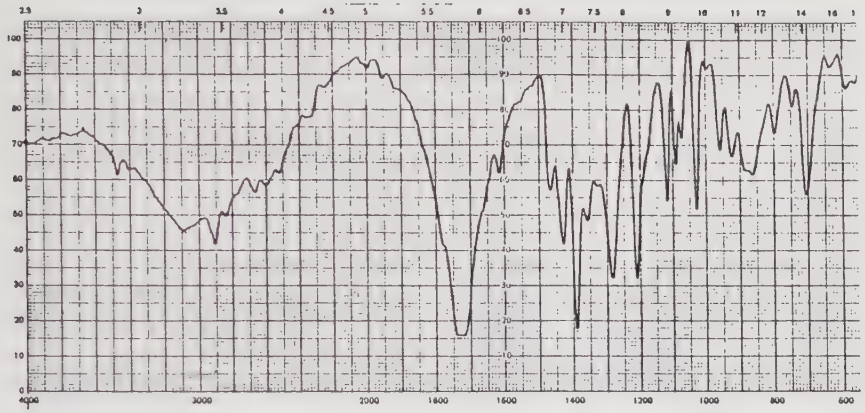

Fig. 41: IR spectrum of 2-phthalimidoglutaric acid
2850 (COOH); 1640-1620 (C=O).

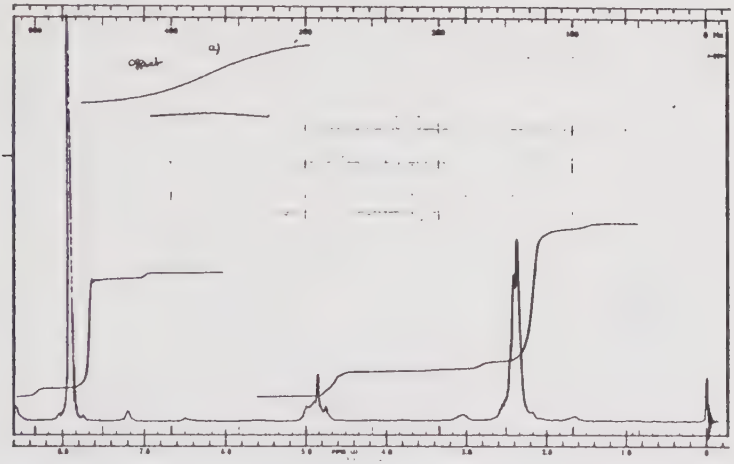

Fig. 42: ^{1}H.n.m.r. of 2-phthalimidoglutaric acid in DMSO-d_6:
2.4 (m, 4H, 2 CH_2); 4.86 (m, 1H, CH); 7.93 (s, 4H,
aromatic); offset (s, 2H, 2 COOH, "a").

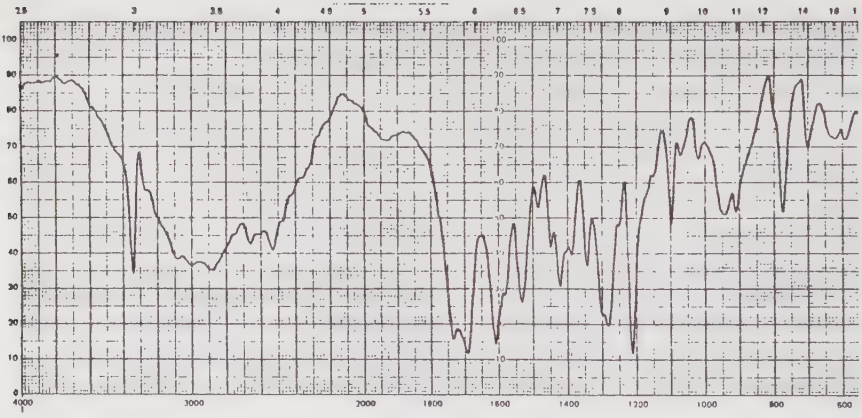

Fig. 43: IR spectrum of 2-(o-carboxybenzamido)glutaric acid 3340 (NH); 2870 (COOH); 1740-1680 (C=O).

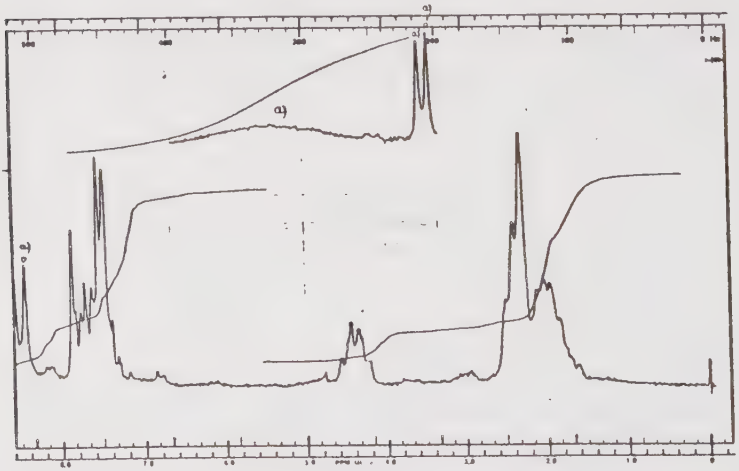

Fig. 44: ^{1}H.n.m.r. of 2-(o-carboxybenzamido)glutaric acid in DMSO-d_6: 2.01 (m, 4H, 2 CH_2); 4.43 (m, 1H, CH); 7.6 (m, 4H, aomatic); 8.5 (d, 1H, NH, "a"); offset (s, 3H, 3 COOH, "a").

5.9. Reduction of a Mixture of Thalidomide Hydrolysis Products According to the Percentage Obtained by Beckmann in Table 1

In a 50-ml double wall flask, equipped as before, 5 ml of bis(4-methylpiperazinyl)aluminium hydride were placed by the use of a syringe. The solution was allowed to cool to 0°C. 35 mg of the mixture of thalidomide hydrolysis products (according to the percentage obtained in urine of man by Beckmann in Table 1) in 5 ml of absolute THF (cooled to 0°C) were added slowly with vigorous stirring. The solution was refluxed for 12 hrs with occasional addition of a few ml of absolute THF during the reflux. Then water (0.2 ml) was added to the solution at 0°C followed by 3 ml of carbonyl-free methanol. Crystals were filtered off and extracted repeatedly with carbonyl-free methanol. The filtrate and extracts were condensed in vacuo to about 5 ml. The residue was analysed for aldehydes content as in 5.3.2.. After 12 hrs of reflux, 48 mg of 2,4-DNPH derivatives were obtained.

5.9.1. Quantitative Measurement of the Colour Developed from the Foregoing Hydrazones

The obtained 48 mg of hydrazones were dissolved in 100 ml of methanolic potassium hydroxide reagent. From this solution samples of 0.25, 0.5, 0.75, 1 and 1.25 ml were taken by the use of a micropipette and each sample was diluted up to 25 ml with carbonyl-free methanol. After standing one hour, the measurements of the absorption of colour started with the lowest concentration up to the highest one. The results obtained are shown in Table 11, Figure 15 and Figure 16.

5.10. Reduction of Thalidomide Hydrolysis Products, Obtained after its Hydrolysis at pH 7.4 and 37°C for 24 hrs at Room Temperature

In a 50-ml round bottomed flask, 49 mg of thalidomide were dissolved in 5 ml of dioxan, then 11 ml of phosphate buffer (pH 7.4) were added. The mixture was left in a water bath at 37°C for 24 hrs. After the end of hydrolysis, the solvent was evaporated in vacuo and the residue was dried at 110°C under vacuum. The flask containing the dried residue was equipped with a P_2O_5 drying tube, stirring bar, dry argon inlet and thermometer. 5 ml of absolute THF were added to the residue and the solution was allowed to cool to 0°C. 7 ml of bis(4-methylpiperazinyl)aluminium hydride reagent were added. The solution was stirred at room temperature, while the flask was well closed after its charging with dry argon. Then water (0.3 ml) was added to the solution at 0°C followed with 4 ml of carbonyl-free methanol. Crystals were filtered off and extracted repeatedly with carbonyl-free methanol. The filtrate and the extracts were condensed in vacuo to about 5 ml. This residue was analysed for aldehydes contents as in 5.3.2.. After two days of stirring at room temperature 40 mg of 2,4-DNPH derivatives were obtained.

5.10.1. Quantitative Measurement of the Colour Developed from the Foregoing Hydrazones

The obtained 40 mg of hydrazones were dissolved in 50 ml of methanolic potassium hydroxide reagent. From this solution samples of 0.2, 0.4, 0.6, 0.8 and 1 ml were taken by the use of a micropipette and each sample was diluted up to 25 ml with carbonyl-free methanol. After standing for one hour, the measurements of the absorption of colour were begun with the lowest concentration up to the highest one. The results are shown in Table 12, Figure 17 and Figure 18.

5.11. Reduction of Thalidomide Hydrolysis Products, Obtained after its Hydrolysis at pH 7.4 and 37°C for 24 hrs, under Reflux

In a 50-ml round bottomed flask, 53.5 mg of thalidomide were dissolved in 5.5 ml dioxan, then 11.5 ml of phosphate buffer (pH 7.4) were added. The mixture was left in a water bath at 37°C for 24 hrs. After the end of hydrolysis, the solvent was evaporated in vacuo and the residue was well dried at 110°C in vacuum. The flask containing the dried residue was equipped with a reflux condenser with P_2O_5 drying tube, stirring bar, dry argon inlet and thermometer. 5 ml of absolute THF were added to the residue and the solution was allowed to cool to 0°C. 7.5 ml of bis(4-methylpiperazinyl)aluminium hydride reagent were added. The solution was refluxed for 12 hrs with occasional addition of a few ml of absolute THF during the reflux. Then water (0.3 ml) was added to the solution at 0°C, followed by 4 ml of carbonyl-free methanol. Crystals were filtered off and extracted repeatedly with carbonyl-free methanol. The filtrate and the extracts were condensed in vacuo to about 5 ml. This residue was analysed for aldehydes content as in 5.3.2.. After 12 hrs of reflux, 80 mg of 2,4-DNPH derivatives were obtained.

5.11.1. Quantitative Measurement of the Colour Developed from the Foregoing Hydrazones

The obtained 80 mg of hydrazones were dissolved in 100 ml of methanolic potassium hydroxide reagent. From this solution samples of 0.2, 0.4, 0.6, 0.8 and 1.0 ml were taken by the use of a micropipette and each sample was diluted up to 25 ml with carbonyl-free methanol. After standing for one hour, the measurements of the absorption of colour was begun with the lowest concentration up to the highest one. The results are shown in Table 13, Figure 19 and Figure 20.

5.12. Reduction of Thalidomide Hydrolysis Products, Obtained after its Hydrolysis at pH 7.4 and 37°C for 4 hrs, under Reflux

In a 50-ml round bottomed flask, 43 mg of thalidomide were dissolved in 4.5 ml dioxan, then 10.5 ml of phosphate buffer (pH 7.4) were added. The mixture was left in a water bath at 37°C for 4 hrs. After the end of hydrolysis, the solvent was evaporated in vacuo and the residue was well dried at 110°C under reduced pressure. The flask containing the dried residue was equipped as before. 5 ml of absolute THF were added to the residue and the solution was allowed to cool to 0°C. 6.2 ml of bis(4-methylpiperazinyl)aluminium hydride reagent were added. The solution was refluxed for 12 hrs with occasional addition of a few ml of absolute THF during the reflux. Then water (0.3 ml) was added to the solution at 0°C followed by 4 ml of carbonyl-free methanol. Crystals were filtered off and extracted repeatedly with carbonyl-free methanol. The filtrate and the extracts were condensed in vacuo to about 5 ml. This residue was analysed for aldehydes content as in 5.3.2.. After 12 hrs of reflux 38.5 mg of 2,4-DNPH derivatives were obtained.

5.12.1. Quantitative Measurement of the Colour Developed from the Foregoing Hydrazones

The obtained 38.5 mg of hyrazones were dissolved in 100 ml of methanolic potassium hydroxide reagent. From this solution samples of 0.5, 1, 1.5, 2 and 2.5 ml were taken and each sample was diluted up to 25 ml with carbonyl-free methanol. After standing for one hour, the measurements of the absorption of colour were begun, starting with the lowest concentration up to the highest one. The results obtained are shown in Table 14, Figure 21 and Figuire 22.

5.13. Reduction of Thalidomide Hydrolysis Products, Obtained after its Hydrolysis at pH 8 and 37°C for 24 hrs, under Reflux

In a 25-ml round bottomed flask, 24 mg of thalidomide were dissolved in 2.5 ml dioxan, then 6 ml of phosphate buffer (pH 8) were added. The mixture was left in a water bath at 37°C for 24 hrs. After the end of hydrolysis, the solvent was evaporated in vacuo and the residue was well dried at 110°C under reduced pressure. The flask containing the dried residue was equipped as before. 5 ml of absolute THF were added to the residue and the solution was allowed to cool to 0°C. 3.5 ml of bis(4-methylpiperazinyl)aluminium hydride were added. The solution was refluxed for 12 hrs with occasional addition of a few ml of absolute THF during the reflux. Then water (0.2 ml) was added to the solution at 0°C followed by 3 ml of carbonyl-free methanol. Crystals were filtered off and extracted repeatedly with carbonyl-free methanol. The filtrate and the extracts were condensed in vacuo to about 5 ml. This residue was analysed for aldehydes content as in 5.3.2.. After 12 hrs of reflux, 36 mg of 2,4-DNPH derivatives were obtained.

5.13.1. Quantitative Measurement of the Colour Developed from the Foregoing Hydrazones

The obtained 36 mg of hydrazones were dissolved in 100 ml of methanolic potassium hydroxide reagent. From this solution samples of 0.5, 1, 1.5, 2 and 2.5 ml were taken and each sample was diluted up to 25 ml with carbonyl-free methanol. After standing for one hour, the measurements of the absorption of colour were begun, starting with the lowest concentration up to the highest one. The results obtained are shown in Table 15, Figure 23 and Figure 24.

5.14. Reduction of Thalidomide Hydrolysis Products, Obtained after its Hydrolysis at pH 8 and 37°C for 4 hrs, under Reflux

In a 25-ml round bottomed flask, 28 mg of thalidomide were dissolved in 3 ml dioxan, then 7 ml of phsophate buffer (pH 8) were added. The mixture was left in a water bath at 37°C for 4 hrs. After that, the solvent was evaporated in vacuo and the residue was well dried at 110°C under reduced pressure. The flask containing the dried residue was equipped as before. 5 ml of absolute THF were added to the residue and the solution was allowed to cool to 0°C. 4 ml of bis(4-methylpiperazinyl)-aluminium hydride were added. The solution was refluxed for 12 hrs with occasional addition of a few ml of absolute THF during the reflux. Then water (0.2 ml) was added to the solution at 0°C followed by 3 ml of carbonyl-free methanol. Crystals were filtered off and extracted repeatedly with carbonyl-free methanol. The filtrate and the extracts were condensed in vacuo to about 5 ml. This residue was analysed for aldehydes content as in 5.3.2.. After 12 hrs of reflux 39.5 mg of 2,4-DNPH derivatives were obtained.

5.14.1. Quantitative Measurement of the Colour Developed from the Foregoing Hydrazones

The obtained 39.5 mg of hydrazones were dissolved in 100 ml of methanolic potassium hydroxide reagent. From this solution samples of 0.5, 1, 1.5, 2 and 2.5 ml were withdrawn and each sample was diluted up to 25 ml with carbonyl-free methanol. After standing for one hour, the measurements of absorption were begun starting with the lowest concentration up to the highest one. The results obtained are shown in Table 16, Figure 25 and Figure 26.

5.15. Reduction of Thalidomide Hydrolysis Products, Obtained after its Hyrdolysis at pH 6 and 37°C for 24 hrs, under Reflux

In a 50-ml round bottomed flask, 38 mg of thalidomide were dissolved in 4 ml of dioxan, then 9 ml of phosphate buffer (pH 6) were added. The mixture was left in a water bath at 37°C for 24 hrs. After the evaporation of the solvent and drying of the residue at 110°C under reduced pressure, the flask containing the well dried residue was equipped as before. 5 ml of absolute THF were added to the residue and the solution was allowed to cool to 0°C. 5.5 ml of bis(4-methylpiperazinyl)-aluminium hydride were added. The solution was refluxed for 12 hrs with occasional addition of a few ml of absolute THF during the reflux. Then water (0.3 ml) was added to the solution at 0°C followed by 4 ml of carbonyl-free methanol. Crystals were filtered off and extracted repeatedly with carbonyl-free methanol. The filtrate and the extracts were condensed in vacuo to about 5 ml. This residue was analysed for aldehydes content as in 5.3.2.. After 12 hrs reflux 25 mg of 2,4-DNPH derivatives were obtained.

5.15.1. Quantitative Measurement of the Colour Developed from the Foregoing Hydrazones

The obtained 25 mg of hydrazones were dissolved in 100 ml of methanolic potassium hydroxide reagent. From this solution samples of 0.5, 1, 1.5, 2 and 2.5 ml were withdrawn and each sample was diluted up to 25 ml with carbonyl-free methanol. After standing for one hour, the measurements were begun with the lowest concentration. The results are shown in Table 17, Figure 27 and Figure 28.

5.16. Applications on Small Weight Quantities of Thalidomide Hydrolysed at Different pH Values and 37°C for Different Times

Six samples of small quantities of thalidomide 1, 2, 3, 4, 5 and 6 were dissolved in 2 ml dioxan. 4 ml of buffer solution (pH 7.4) were added to the samples 1, 2 and 3; 4 ml of buffer solution (pH 8) were added to the samples 4 and 5; and 4 ml of buffer solution (pH 6) was added to sample 6.
The samples 1, 2, 4 and 6 were left in a water bath at 37°C for 24 hrs; the samples 3 and 5 were left in a water bath at 37°C for 4 hrs. After the end of hydrolysis, the solvents were evaporated in vacuo and the residues were well dried at 110°C under reduced pressure. 5 ml of absolute THF were added to each residue and each solution was allowed to cool to 0°C. 4 ml of bis(4-methylpiperazinyl)aluminium hydride reagent were added to each sample. In sample 1 the solution was stirred at room temperature for 2 days, while the samples 2, 3, 4, 5 and 6 were refluxed for 12 hrs with occasional addition of a few ml of absolute THF during the reflux. Then water (0.2 ml) was added to the solutions at 0°C, followed by 3 ml of carbonyl-free methanol. Crystals were filtered off and extracted repeatedly with carbonyl-free methanol. The filtrates and the extracts were diluted volumetrically up to 100 ml with carbonyl-free methanol.

5.16.1. Method for Unknown Trace Quantities of Aldehydes

To 1.0 ml of the foregoing unknown solutions in carbonyl-free methanol, 1.0 ml of 2,4-dinitrophenylhydrazine reagent and 0.1 ml of concentrated hydrochloric acid were added. The tubes were stoppered loosely and heated in a water bath (100°C) for 5 minutes. After cooling 0.5 ml of the potassium hydroxide solution were added to make the volume 2.5 ml. The almost black solutions that resulted, rapidly cleared to the characteristic colour. This colour was well developed after standing

for one hour. Simultaneously a blank determination was made, using 1.0 ml of carbonyl-free methanol in place of the sample. The optical density of the solutions was determined.

From the constructed graphs (calibration curves) and their regression equations the obtained absorption was replaced in each equation to obtain the concentration of the hydrazones in 25 ml solvent. The obtained concentration was multiplied by 10 to obtain the actual quantity in each unknown sample and this concentration was related to the amount of thalidomide which was present as unknown.

The results obtained are shown in Table 19.

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